Combined genomic and expression microarray analysis of paediatric astrocytoma

Presented by

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ABSTRACT

Although astrocytomas account for 40% of brain tumours in children, little is known about the genetics of these paediatric tumours. Indeed, 85% of low-grade (WHO grade I and II) and 50% of high-grade (WHO grade III and IV) paediatric astrocytoma have a normal karyotype. The aim of this study was to identify non-random genetic aberrations in different grades of paediatric astrocytoma at both the genomic and expression levels. Affymetrix genechip technology and array comparative genomic hybridisation (aCGH) have been used to generate gene expression profiles of 35 paediatric astrocytoma short-term cell cultures of all grades and 19 pilocytic (PA, grade I) biopsies and identify copy number alterations (CNA) in 32 paediatric astrocytoma short-term cell cultures of all grades and 11 pilocytic biopsies.

The PAI biopsy samples have a distinct expression profile compared to normal brain with 1844 genes being differentially expressed in all samples. The KEGG pathway most influenced by these genes is antigen processing and presentation, with the majority of genes being up-regulated. Addition pathways altered include PI3K signalling and MAPK signalling. Only single clone CNAs were detected in PAI including alterations at 1p36.32-p36.3, 14q12 and 22q33.33 which were lost or gained in the majority of samples. The clone at 14q12 is located in a region of large-scale copy number alteration (LCV). Alterations at this site have been linked to increased risk of paediatric solid tumour development. No known genes are located within the clone site. However, FOXG1B is adjacent to the clone region and is significantly down-regulated in PAI compared to normal brain.

Hierarchical clustering of the short-term cultures according to expression profile similarity demonstrated that paediatric astrocytoma can be grouped into low and high-grade tumours by molecular signature. Furthermore, approximately half of paediatric glioblastoma multiforme (GBMIV, grade IV) clustered with 7 adult GBMIV cultures, suggesting that some paediatric GBMIV are genotypically similar to those arising in adults. KEGG pathways influenced by differential gene expression include Wnt signalling and the cell cycle pathway, with the finding that same pathways are being disrupted to varying extents in low and high-grade paediatric astrocytoma. The frequency of CNAs was similar to those previously reported, with gain of all or part of chromosome 7 as the most common alteration. Correlations between gene expression and CNAs were also identified in the short-term cultures.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAIII	-	anaplastic astrocytoma, grade III
aCGH	-	array comparative genomic hybridisation
AC	-	adenocarcinomas
ALT	-	alternative lengthening of telomeres
Ang-1	-	angiopoietin-1
Ang-2	-	angiopoietin-2
APS	-	ammonium sulphate
BMPs	-	bone morphogenetic proteins
CBTRUS	-	Central Brain Tumour Registry of the United States
CML	-	chronic myeloid leukaemia
CNA	-	copy number alteration
CNS	-	central nervous system
CSF	-	cerebrospinal fluid
CTCL	-	cutaneous T-cell lymphoma
CT	-	computed tomography
DAII	-	diffuse astrocytoma, grade II
DMSO	-	dimethylsulphoxide
DNA	-	deoxyribonucleic acid
EGFR	-	epidermal growth factor receptor
FCS	-	foetal calf serum
FGF	-	fibroblast growth factor
FISH	-	fluorescent *in situ* hybridisation
GA	-	gemistocytic astrocytoma
GBMIV	-	glioblastoma multiforme, grade IV
GFAP	-	glial fibrillary acidic protein
GO	-	gene ontology
GPI	-	glycophosphatidylinositol
HATs	-	histone acetyltansferases
HDACs	-	histone deacetylases
H&E	-	hematoxylin-eosin stain
HIF-1	-	hypoxia-inducible factor-1
HMTs	-	histone methyltransferases
HSC	-	human Schwann cells
ID	-	inflammatory dermatoses
KEGG	-	Kyoto Encyclopaedia of Genes and Genomes
LCVs	-	large scale copy number variations
LOH	-	loss of heterozygosity
LSCC	-	laryngeal squamous cell carcinoma
5mC	-	5-methylcytosine
MAPK	-	mitogen activated protein kinase
MBP	-	myelin basic protein
MCM	-	mini chromosome maintenance
MF	-	mycosis fungoides
m-FISH	-	multiplex fluorescent *in situ* hybridisation
MGMT	-	methylguanine DNA methyl transferase
miRNAs	-	microRNAs
MPNST	-	malignant peripheral nerve sheath tumours
MRI	-	magnetic resonance imaging

mRNA	-	messenger ribonucleic acid
MSD	-	modified standard deviation
MSP	-	methylation specific PCR
NaOAc	-	sodium acetate
ncRNA	-	non-coding RNA
NCBI	-	National Centre for Biotechnology Information
NF1	-	neurofibromatosis type 1
NSCLC	-	non-small cell lung carcinoma tissues
OS	-	overall survival
OSCC	-	oral squamous-cell carcinoma
OSE	-	ovarian surface epithelium
PAI	-	pilocytic astrocytoma, grade I
PDGFα	-	platelet derived growth factor-alpha
PDGFRα	-	platelet derived growth factor receptor-alpha
PFS	-	progression-free survival
PI3K	-	phosphatidylinositol signalling system
PIP2	-	phosphatidylinositol-4,5-diphosphate
PIP3	-	phosphatidylinositol-3,4,5-triphosphate
PXA	-	pleomorphic xanthoastrocytoma
Q-PCR	-	quantitative real-time polymerase chain reaction
RNA	-	ribonucleic acid
RT	-	radiotherapy
RT-PCR	-	reverse transcriptase polymerase chain reaction
siRNAs	-	small interfering RNAs
SCC	-	squamous cell carcinomas
SCLC	-	small cell lung carcinoma
SD	-	standard deviation
SNP	-	single nucleotide polymorphism
TAE	-	1X tris-acetate
uPAR	-	urokinase plasminogen activator receptor
VEGF	-	vascular endothelial growth factor
VEGFR	-	vascular endothelial growth factor receptor
WHO	-	World Health Organisation
WO	-	whole ovary

CHAPTER 1

INTRODUCTION

The initial goal of this study was to identify differentially expressed genes in different grades of malignancy of paediatric astrocytoma. As with the majority of studies, this study has evolved to include other aspects of brain tumour research such as the use of short-term cell cultures as a tool in the investigation of tumour cells, the use of normal controls in microarray experiments, the current and previous World Health Organisation (WHO) grading scheme, and chromosome alterations and methylation as mechanisms associated with tumour development and differential gene expression. The introduction of this thesis will focus on paediatric astrocytoma but where appropriate will refer to adult astrocytoma.

Astrocytoma histological classification and clinical parameters

Paediatric and adult astrocytomas are classified by WHO guidelines into four grades of malignancy according to histological criteria (Table 1.1). These criteria include nuclear atypia (enlarged, cigar-shaped or irregular hyperchromatic nuclei), mitosis, microvascular proliferation and/or necrosis. All astrocytomas consist of cells with phenotypical characteristics of astrocytes, although the true cell of origin of these tumours is unknown (Ichimura et al., 2004).

Table 1.1 Astrocytoma tumour sub-groups and grades according to WHO guidelines. Adapted from (Louis et al., 2007a).

Astrocytoma	WHO Malignancy Grade
Pilocytic Astrocytoma	I
Pilomyxoid	II
Pleomorphic Xanthoastrocytoma	II
Diffuse Astrocytoma *Fibrillary* *Protoplasmic* *Gemistocytic*	II
Anaplastic Astrocytoma	III
Glioblastoma Multiforme *Giant Cell Glioblastoma* *Gliosarcoma*	IV

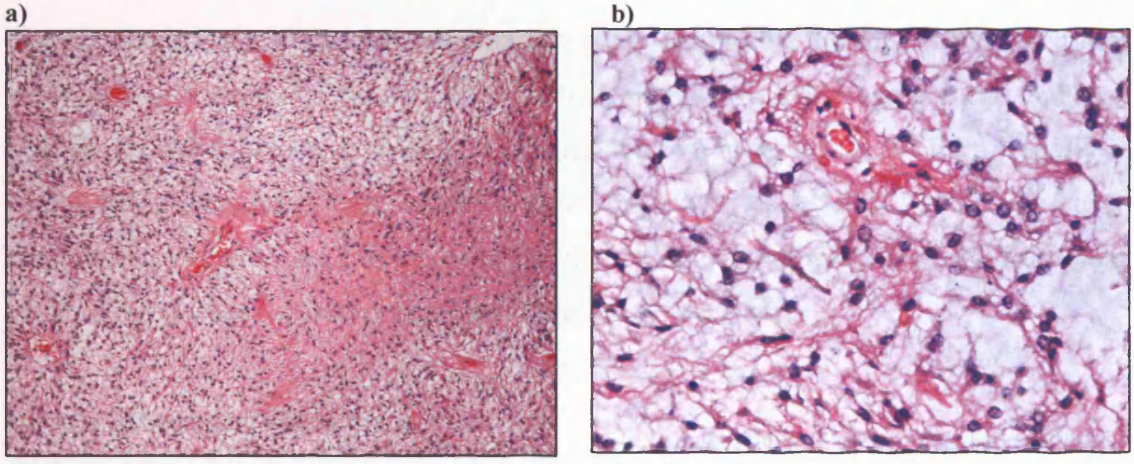
This report will use the terminology and definitions of the WHO classification of 2007 throughout (Louis et al., 2007a).

Pilocytic astrocytoma

Pilocytic astrocytoma are WHO grade I tumours (PAI). This tumour is the most frequent astrocytoma in children, commonly arising in the first two decades of life. PAI can arise in many regions of the central nervous system (CNS) including the cerebellum, cerebral hemispheres, thalamus and basal ganglia, optic nerve and chiasm, brain stem and spinal cord. These tumours are slow growing masses that can stabilise or regress but are rarely fatal. PAI of the hypothalamus or brain stem can be associated with poorer prognosis, prolonged clinical course and lesion recurrence. It has also been noted that a small number of both adult and paediatric PAI recur and in very rare cases transform into cellular mitotically active neoplasms (Rodriguez et al., 1990; Alshail et al., 1997; Broniscer et al., 2007).

The cells of PAI are pilocytes or hair cells that have long extensions and aggregate together forming a fibrillary background. The uniform nuclei of these cells appear elongated and the polar cells within the tumour are usually GFAP positive. Rosenthal fibres are common in dense regions of the lesion. Furthermore, microcysts are common and are usually uniformly spaced (Louis et al., 2007a). The typical biphasic pattern of these tumours can be seen in Figures 1.1a and 1.1b.

Figure 1.1 H&E stained paediatric pilocytic astrocytoma, a) x60 and b) x400 respectively.



All H&E stained pictures in this thesis are credited to Dr T. J. Jacques. These pictures illustrate the typical biphasic pattern of paediatric PAI.

Pilomyxoid

This tumour is closely related to a pilocytic astrocytoma and corresponds to a grade II astrocytoma that is more aggressive, with local recurrence and cerebrospinal spread occurring more frequently. This tumour is typically seen in very young children (mean age 10 months), although it can occur in older children and commonly presents in hypothalamic/chiasmatic regions.

Pilomyxoid tumours have a prominent mucoid matrix consisting of bipolar cells with an angiocentric monomorphous arrangement. Rosenthal fibres and eosinophilic granular bodies are not commonly found in this tumour. However, the cells usually demonstrate strong reactivity for GFAP.

Pleomorphic xanthoastrocytoma

These grade II tumours account for less than 1% of all astrocytoma and have a favourable prognosis. The lesions occur most commonly in children and young adults, with most developing before 18 years of age. Pleomorphic xanthoastrocytoma (PXA) are often located in the cerebral hemispheres involving the meninges, preferentially in the temporal lobe. However, these tumours have also been found in the cerebellum and spinal cord.

The histopathology of this astrocytoma has many forms (pleomorphic). Spindly fibres are mixed with mono- or multinucleated cells, which themselves vary in shape and size. Mitotic activity is clearly visible, with progression to a more malignant form, usually in adults, being associated with increased mitotic activity (Kepes et al., 1989). The term xanthoastrocytoma is used to describe the intracellular accumulation of lipid droplets, which often pushes the intracellular structures to the periphery.

Diffuse infiltrating astrocytoma

Diffuse astrocytoma

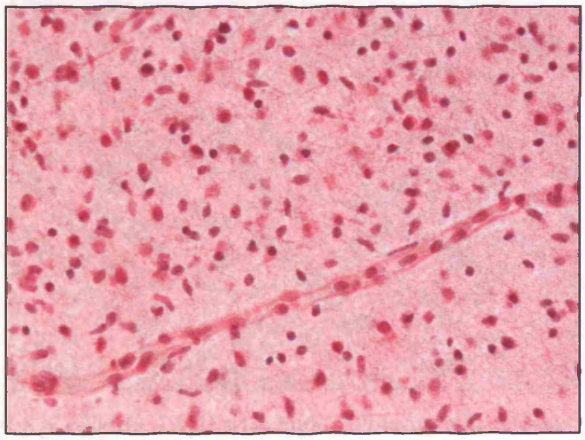
Diffuse astrocytoma are classified as WHO grade II (DAII) and nuclear atypia is the only criterion required for this classification. These tumours have a high degree of cellular differentiation, are slow growing, usually supratentorial and infiltrate neighbouring brain structures (Perry, 2003). Only 10% of cases occur in patients below

20 years of age. In adults, DAII commonly progress to a more malignant tumour (AAIII or GBMIV), but this is rarely seen in paediatric patients.

Several DAII sub-types are recognised including fibrillary, gemistocytic and protoplasmic astrocytoma. Fibrillary astrocytoma are the most common, consisting of fibrillary cells that show limited nuclear atypia and often resemble astrocytes. A scarce and poorly visible cytoplasm gives the appearance of 'naked nuclei'. In addition to brain infiltration, these tumours may also contain multiple microcysts. GFAP expression is seen throughout the tumour, but not in all cells. Figure 1.2 illustrates the low cellularity and 'naked nuclei' associated with DAII.

Gemistocytic astrocytoma are composed of gemistocytic neoplastic cells which often have a plump, glassy, eosinophilic cell body appearance and are randomly distributed within a coarse fibrillary network. This variant progresses more rapidly to a higher grade tumour in adults (Watanabe et al., 1997b; Reis et al., 2001). The expression of GFAP is consistently seen in these cells.

Figure 1.2 H&E stained paediatric fibrillary diffuse astrocytoma, x150.



This picture illustrates a paediatric DAII with minimal nuclear atypia and low cellularity.

Protoplasmic astrocytoma are composed of cells with an astrocytic phenotype. The cell bodies are small with a low content of glial filaments, the nuclei are round to oval in shape and microcysts are commonly seen. GFAP staining is scarce. This variant is rare, with few DAII being classed as pure protoplasmic DAII (Prayson and Estes, 1995). In the opinion of the pathologists at Great Ormond Street Hospital, protoplasmic

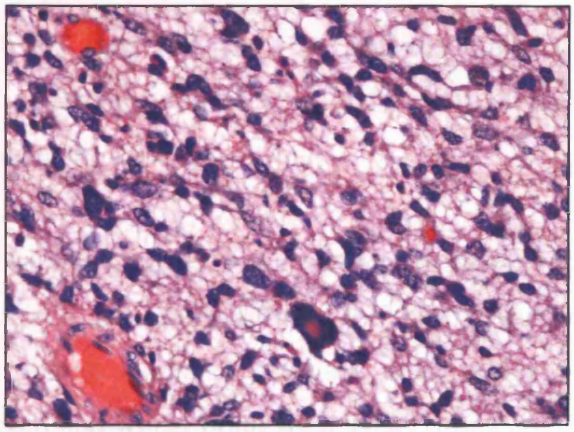
astrocytoma may not exist as an astrocytoma subtype, but rather a feature of some tumours.

Anaplastic astrocytoma

Anaplastic astrocytoma are classified as WHO grade III (AAIII). Nuclear atypia and mitotic activity characterise these astrocytomas which have focal or dispersed anaplasia and show increased proliferation. Very few cases are seen below the age of 20 years. The common location of this tumour is very similar to that of other DAII, preferentially in the cerebral hemispheres.

Anaplastic astrocytoma have increased cellularity and complex nuclear morphology (Figure 1.3). The nucleus may appear enlarged and vary in shape or chromatin distribution. Multinucleation can also be seen, along with normal and abnormal mitoses. Inconsistent GFAP staining is often found in this tumour and gemistocytic cells may be present in small numbers.

Figure 1.3 H&E stained paediatric anaplastic astrocytoma, x400.

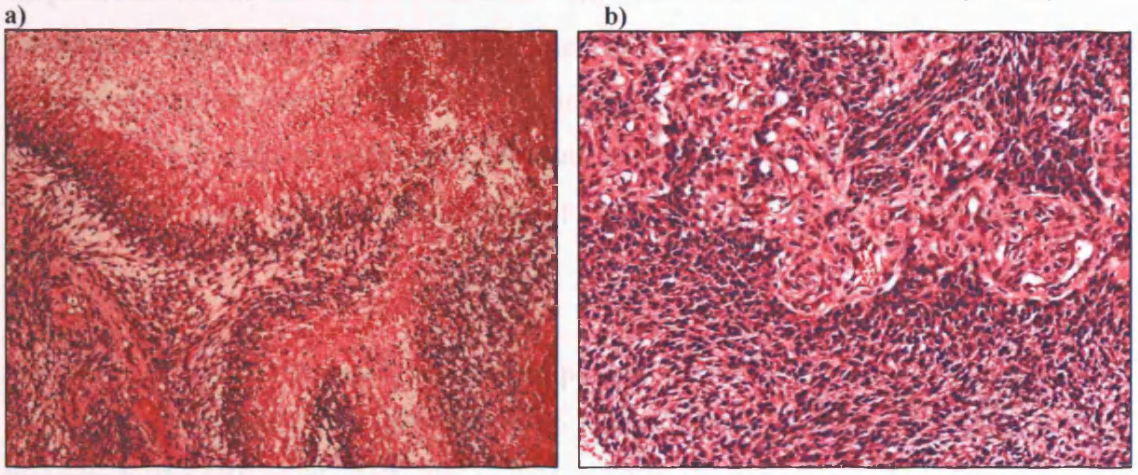


This picture illustrates a paediatric AAIII with high cellularity and cellular and nuclear anaplasia.

Glioblastoma multiforme

Glioblastoma multiforme (GBMIV) are the most malignant astrocytoma and are classified as WHO grade IV. Any three of the four histological criteria, nuclear atypia, mitosis, microvascular proliferation and/or necrosis are required to diagnose this tumour, which is often composed of poorly differentiated neoplastic cells with an astrocytic phenotype (Figure 1.4).

Figure 1.4 H&E stained paediatric glioblastoma multiforme, a) x50 and b) x100 respectively.



These pictures illustrate a paediatric GBMIV with paeudopalisading necrosis (a) and microvascular proliferation (b).

As a group, the peak incidence of adult GBMIV is between 45 and 70 years of age. However, GBMIV may develop *de novo* (primary GBMIV) or from low-grade tumours (secondary GBMIV). Primary GBMIV are more common, manifesting after a short clinical history, usually during or after the sixth decade of life. Secondary GBMIV progress from low-grade tumours over a period of months or years and usually occur in the in the fourth decade of life (Kleihues and Ohgaki, 1999). Less than 3% occur in the first two decades of life, but this may be underestimated as resections are not necessarily carried out when the tumours are found in difficult locations in children, e.g. in the brain stem (Louis et al., 2007a).

GBMIV are most commonly located in the cerebral hemispheres, preferentially in the frontotemporal and parietal area. Infiltration of the tumour can reach the basal ganglia or contralateral hemispheres. On rare occasions, these tumours can be found in the cerebellum or spinal cord and whilst brain stem astrocytoma are less common in adults, they affect between 3-9% of children diagnosed with astrocytoma (Pollack, 1999a; Louis et al., 2007a).

Giant cell GBMIV are composed of multinucleated giant cells with a prominent stromal reticulin network. This is a rare variant, accounting for less than 5% of GBMIV. Furthermore, this characteristic may exist as a tumour feature rather than an overall diagnosis. The staining of GFAP is also sporadic in these cells.

Gliosarcoma can be described as a GBMIV with sarcomatous components. Areas within the tumour often show gliomatous or mesenchymal differentiation, with glial regions displaying various degrees of GFAP staining and anaplasia. Spindle cells in long bundles and herringbone patterns of fibrosarcoma characterise the sarcomatous regions of the tumour. This variant accounts for approximately 2% of all GBMIV (Louis et al., 2007a).

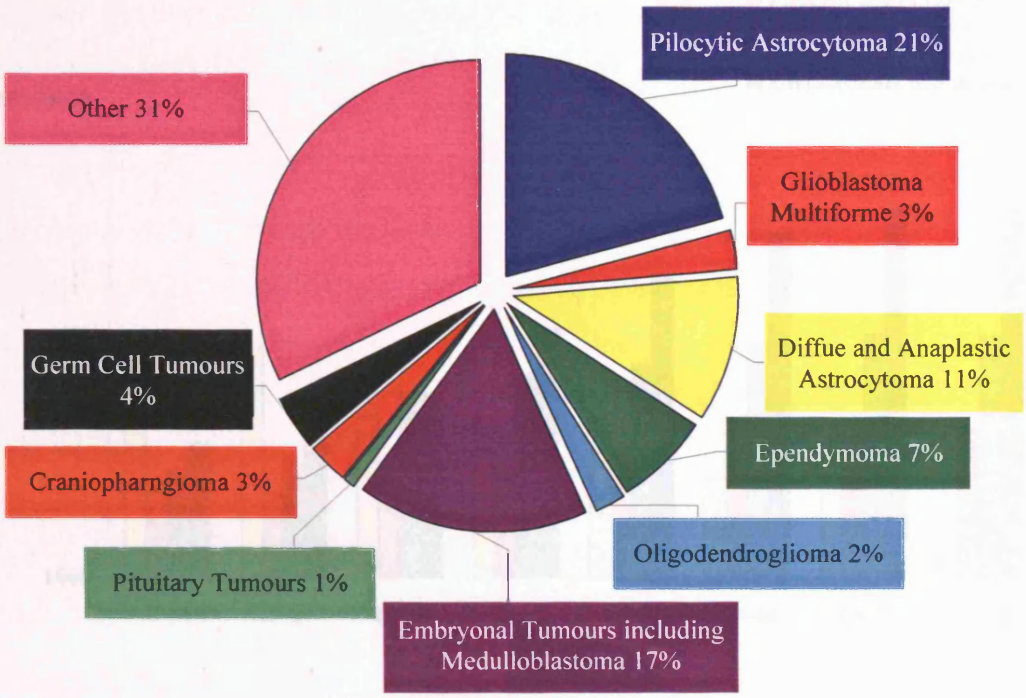
Incidence of paediatric astrocytoma and patient survival

It is difficult to determine the precise incidence and mortality of childhood brain tumours in the UK, particularly the various grades of astrocytoma. This is because the incidence and survival rate of astrocytoma grades in children below 15 years of age are not individually documented. The Office for National Statistics (www.statistics.gov) documents malignant neoplasms of the brain as a group and does not clarify the incidence of individual tumours or grades. Furthermore, the information provided by Cancer Research UK (www.cancerresearch.uk.org) regarding brain tumour incidence, sources the information provided by The Office for National Statistics. The Childhood Cancer Research Group also referenced by Cancer Research UK, combines astrocytoma incidence and survival data providing information that reflects astrocytoma tumours as a group and not PAI, DAII, AAIII and GBMIV as individual grades.

The Central Brain Tumour Registry of the United States (CBTRUS) provides an alternative source of childhood brain tumour incidence and mortality rates that is documented by grade. Between 1998 and 2002, the CBTRUS collected incidence and survival data for all newly diagnosed cases of non-malignant (benign and uncertain) and malignant primary brain and central nervous system tumours, from eighteen collaborating state cancer registries. This data was complied into the "2005-2006 Primary Brain Tumours in the United States Statistical Report".

Brain tumours are the second most common malignancy among children after leukaemia, but are the most common form of solid tumour. The most frequent histologies in the 0-14 year age group include PAI and embryonal tumours (medulloblastomas), which account for 21% and 17% of cases respectively (Figure 1.5).

Figure 1.5 The distribution of brain tumours occurring in children between 0-14 years of age.

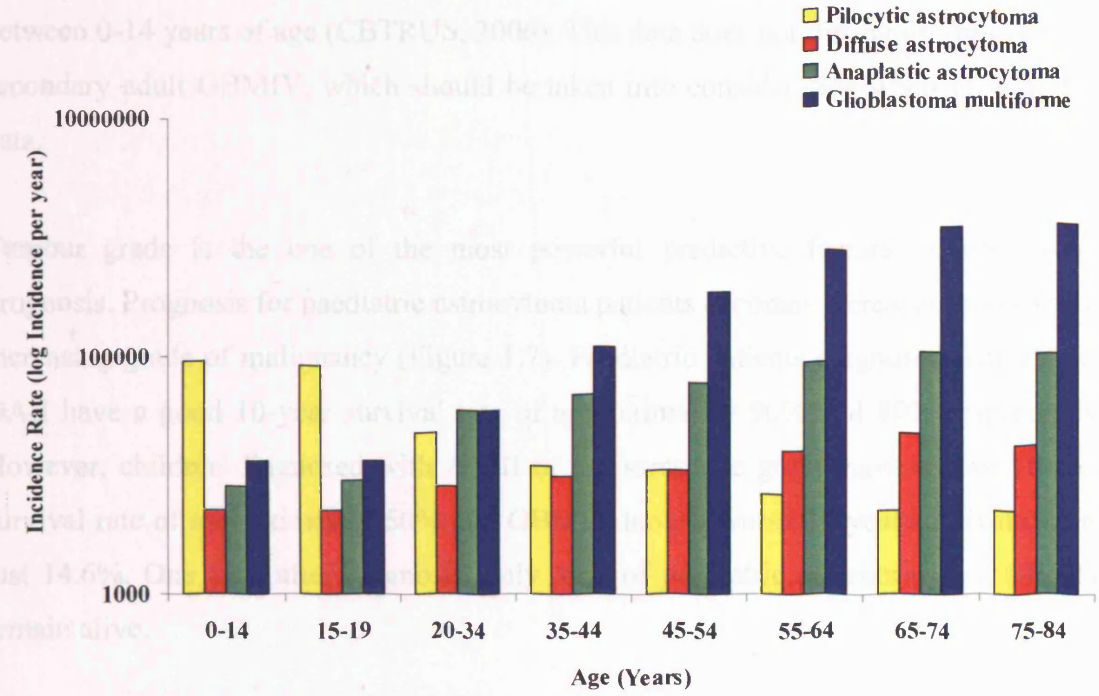


Adapted from the Central Brain Tumour Registry of the United States Report 2005-2006 (CBTRUS, 2006).

The incidence of PAI peaks among children between 5-9 years of age with the broad glioma category accounting for 44% of tumours in children 0-14 years of age (CBTRUS, 2006).

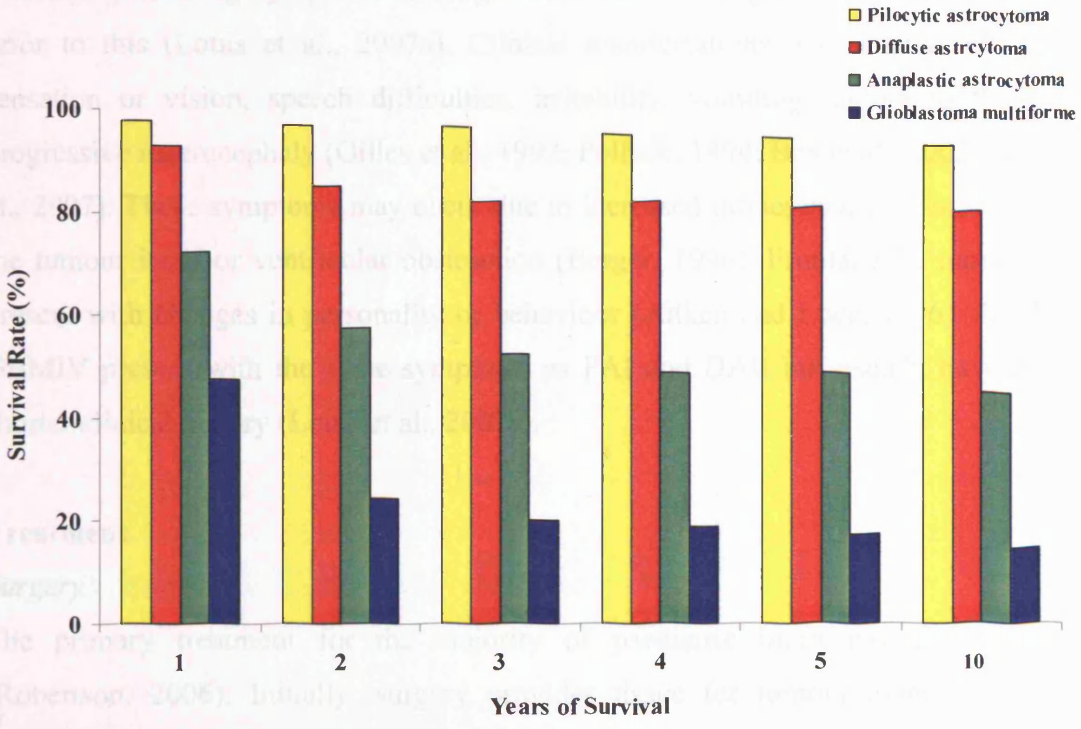
The incidence of astrocytoma varies greatly between different age groups (Figure 1.6). The incidence rate of PAI in children between 0-14 years of age is 0.92 per 100,000 population/year. This compares to the incidence of PAI in patients over 55 years of age of 0.06 per 100,000 population/year. DAII have a low incidence rate between 0-14 years of age of 0.05 per 100,000 population/year. This remains constant between 15-19 years of age but rises continuously with age, peaking between the ages of 65-74 years at a rate of 0.23 per 100,000 population/year. AAIII shows a similar pattern to DAII occurring at a rate of 0.08 per 100,000 population/year between the ages of 0-14 years and peaking between the ages of 65-74 years at a rate of 1.12 per 100,000 population/year. Moreover, GBMIV are most common in patients over 60 years of age with a peak incident rate of 13.45 per 100,000 population/year between 75-84 years of age.

Figure 1.6 The incidence of astrocytoma in the US population according to age.



Adapted from the Central Brain Tumour Registry of the United States Report 2005-2006 (CBTRUS, 2006).

Figure 1.7 The survival rates of children between the ages of 0-14 years diagnosed with an astrocytoma.



Adapted from the Central Brain Tumour Registry of the United States Report 2005-2006 (CBTRUS, 2006).

This compares to an incidence rate of 0.13 per 100,000 population/year in patients between 0-14 years of age (CBTRUS, 2006). This data does not distinguish primary and secondary adult GBMIV, which should be taken into consideration when viewing this data.

Tumour grade is the one of the most powerful predictive factors in astrocytoma prognosis. Prognosis for paediatric astrocytoma patients becomes increasingly poor with increasing grade of malignancy (Figure 1.7). Paediatric patients diagnosed with PAI or DAII have a good 10-year survival rate of approximately 90% and 80% respectively. However, children diagnosed with AAIII of the same age group have a poor 10-year survival rate of approximately 50% and GBMIV have a worse 10-year survival rate of just 14.6%. One year after diagnosis, only 50% of paediatric patients with a GBMIV remain alive.

Clinical manifestations of paediatric astrocytoma

In paediatric patients, the symptoms of astrocytoma are similar to those seen with other solid brain tumours and depend on tumour location and rate of growth. Seizures are a common presenting symptom, although more subtle changes in a patient may occur prior to this (Louis et al., 2007a). Clinical manifestations may include changes in sensation or vision, speech difficulties, irritability, vomiting, failure to thrive and progressive macrocephaly (Gilles et al., 1992; Pollack, 1994; Bos et al., 2002; Dorner et al., 2007). These symptoms may occur due to increased intracranial pressure caused by the tumour itself or ventricular obstruction (Berger, 1996). Frontal lobe tumours may present with changes in personality or behaviour (Aitken and Luce, 1996). AAIII and GBMIV present with the same symptoms as PAI and DAII but usually have a much shorter clinical history (Louis et al., 2007a).

Treatment

Surgery

The primary treatment for the majority of paediatric brain tumours is surgery (Robertson, 2006). Initially, surgery provides tissue for tumour diagnosis, reduces tumour burden and may also restore the cerebrospinal fluid (CSF) pathway (Tomita, 1998; Pollack, 1999b; Robertson, 2006). It has also been suggested that surgery may

enhance tumour response to adjuvant irradiation and/or chemotherapy; the smaller the tumour the greater the response (Tomita, 1998).

Complete resection relies on a well-defined margin between tumour edge and normal brain, with tumour infiltration into normal tissue limiting surgery success (Pollack, 1999b). In low-grade paediatric astrocytoma, particularly PAI, where infiltration rate is low, and a defined tumour edge can often be seen; complete resection is possible, with a high success rate. High-grade paediatric astrocytoma show increased infiltration and poorly defined tumour edge, therefore only a partial or majority removal of this tumour is usually possible (Pollack, 1999b; Robertson, 2006). Specific paediatric brain tumours, such as diffuse brain stem astrocytoma, are often unresectable, consequently diagnosis is carried out using MRI appearance and in certain cases biopsy alone (Albright et al., 1993).

The extent of paediatric tumour resection has been improved in recent years with the aid of new radiographical and electrophysiological methods (Robertson, 2006). Functional MRI allows examination of tumour proximity to functional brain regions. Image guided stereotactic techniques are now used during surgery to indicate tumour relationships with surrounding brain structures. Electrophysiological monitoring and awake craniotomy is also used during surgery to monitor the extent of tumour resection, reducing neurological morbidity (Rezai et al., 1997; Samdani et al., 2005). These techniques provide the surgeon with a 'map' of the tumour before and during surgery.

Data from clinical and retrospective studies suggests that the extent of astrocytoma resection (all grades of malignancy) in paediatric patients has a significant positive impact on patient survival, although this has never been proven prospectively. All patients who undergo a complete tumour resection, regardless of grade, show a significant increase in 5-year survival compared to patients that undergo an incomplete or partial resection (Wisoff et al., 1998; Finlay and Wisoff, 1999; Fisher et al., 2001).

Radiation therapy

When a complete tumour resection is not possible, radiotherapy (RT) is the primary or adjuvant treatment for paediatric patients. However, RT is associated with several disadvantages and side effects including, cognitive impairment caused by RT damage to

the CNS, vasculopathies, stroke and neuroendocrine deficits. Radiosensitivity is also considerably higher in children below 2 years of age as the CNS is still developing. The long term effects of irradiating such young children include lower IQ, estimated at 25 points per whole body irradiation, smaller stature and endocrine problems including damage to the thalamus and pituitary gland (Duffner et al., 1986; Robertson, 2006). The Paediatric Oncology Group completed a phase II clinical study in which postoperative chemotherapy was given to 198 paediatric malignant brain tumour patients under 36 months of age until the age of 4 years or disease progression, in order to delay radiation to the developing brain. The study concluded that chemotherapy was an effective primary postoperative treatment for malignant brain tumours in young children, achieving disease control for one or two years in some patients. In patients who had total surgical resection or a complete response to chemotherapy, subsequent radiation therapy was not needed (Duffner et al., 1993). RT is often delayed in this age group but remains the adjuvant treatment in older children for all grades of astrocytoma that have recurred or show extensive growth (Pollack et al., 1995; Robertson, 2006).

The use of RT in paediatric oncology is a balance between the effects on tumour cells versus normal tissue within the irradiated volume, particularly in high-grade infiltrative tumours. RT is usually administered at a cumulative dose over several weeks to a target tumour volume, as normal tissue can tolerate several small doses of irradiation much better than one large dose (Larson et al., 1993; Swift, 2002). Advances in this field have been possible due to technology which administers the maximum therapeutic dose to a tumour mass whilst minimising the dose to surrounding normal brain. Three-dimensional conformal RT and intensity-modulated RT have been possible through advancements in computer modelling and RT delivery via multiple portals (Swift, 2002). Gamma knife and stereotactic radiosurgery are precise RT approaches used to treat paediatric tumours that require imaging (CT and MRI) and head immobilisation. A single large fraction of radiation is delivered to a discrete volume of tumour with accuracy using a large number of intersecting beams. These methods are used to treat small, well defined, unresectable tumours, in regions where possible damage to normal tissue is acceptable (Kida et al., 2000; Hodgson et al., 2001; Suh and Barnett, 2003; Marcus et al., 2005).

Chemotherapy

Chemotherapy was first used to treat paediatric astrocytoma in the 1970s, when vincristine was shown to have a significant effect on recurrent low and high-grade astrocytoma with minimal toxicity (Rosenstock et al., 1976). The use of chemotherapeutic agents alone, in combination and combined with RT have significantly improved outcome in children with astrocytoma (Robertson, 2006).

Several studies have investigated the treatment of high-grade astrocytoma in children with chemotherapy and RT in various combinations. A clinical trial carried out by the Society of Pediatric Oncology in 1991 reported the outcome of pre-irradiation chemotherapy for high-grade paediatric astrocytoma. Patients received maintenance chemotherapy involving lomustine, vincristine and cisplatin (irradiation followed by chemotherapy) or sandwich chemotherapy (chemotherapy followed by irradiation) involving ifosfamide, etoposide, methotrexate, cisplatin and cytosine arabinoside. Patients who had extensive chemotherapy, via the sandwich arm of the study, had a better overall survival (median 5.2 years) compared to those patients in the maintenance arm (median 1.9 years). However, the extent of tumour resection was the most significant prognostic factor. Median survival was 5.2 years for patients who underwent a greater than 90% tumour resection, compared to 1.3 years for patients who had an incomplete tumour resection. This study revealed that intensive chemotherapy prior to RT, increases survival rates in patients with high-grade astrocytoma that underwent total resection (Wolff et al., 2002a). A further phase I clinical study carried out by the Society of Pediatric Oncology also established that sandwich chemotherapy in paediatric patients with high-grade astrocytoma increased survival compared to surgery and RT alone (Wolff et al., 2002b).

A phase II study of different high-dose chemotherapy regimens prior to RT suggests that survival rate is increased in paediatric patients with high-grade astrocytoma receiving chemotherapy, but that no specific combination of agents provides further advantage. Three chemotherapeutic regimens were used; carboplatin/etoposide (regimen A), ifosfamide/etoposide (regimen B) and cyclophosphamide/etoposide (regimen C) (MacDonald et al., 2005).

Furthermore, in a phase I study, carboplatin, an alkylating agent with reduced toxicity, was shown to increase survival in paediatric low-grade astrocytoma patients when used as a single agent, or in combination with vincristine (Packer et al., 1993).The Society of Pediatric Oncology and Hematology showed that carboplatin and vincristine chemotherapy could effectively defer RT in a treatment strategy for low-grade chiasmatic-hypothalamic astrocytoma in children and adolescents. In this phase II clinical study, children under 1 year did significantly worse than older children (Gnekow et al., 2004). Furthermore, a phase II trial showed that chemotherapeutic treatment with ifosfamide, carboplatin and etoposide combined with RT increased patient survival and reduce tumour size in patients with AAIII and GBMIV (Lopez-Aguilar et al., 2003).

The use of many individual chemotherapeutic agents has been investigated in the treatment of paediatric astrocytoma. Temozolomide is an imidazotetrazine derivative (alkylating agent) that has low toxicity and has been shown to increase survival in adult patients with GBMIV. Resistance to this agent has been found in tumours of patients that express MGMT (methylguanine DNA methyl transferase), a DNA repair protein. This has been important in identifying patients that will respond to temozolomide treatment (Friedman et al., 2000; Hegi et al., 2005). Temozolomide has been investigated as a treatment for high-grade astrocytoma and unfavourable low-grade astrocytoma in children. Boniscer et al. (2006) evaluated the efficacy of temozolomide alone given after RT in a multi-institutional study involving 31 paediatric patients. The study concluded that temozolomide treatment after RT did not increase patient survival. However, this may have been influenced by variations in patient prognostic factors such as tumour location and patient age at diagnosis. Furthermore, *MGMT* methylation status was not investigated in this study. Gururangan et al. (2007) carried out a phase II study of temozolomide in 32 children with recurrent low-grade glioma. Disease stabilisation was possible in more than 50% of patients for a prolonged period, without significant toxicity. A retrospective study by Donson et al. (2007) concluded that *MGMT* promoter methylation in paediatric GBMIV correlates with a survival benefit and sensitivity to temozolomide.

The blood-brain barrier impermeability limits the efficacy of chemotherapy, as does the over-expression of multidrug resistance proteins in tumours and/or endothelial cells.

Despite high response rates, development of intra-arterial chemotherapy remains limited because of frequent acute brain and eye toxicity. High-dose intravenous chemotherapy rescued by autologous hemopoietic stem cell transplantation is an alternative. This approach may increase drug delivery through the blood-brain barrier, whilst overcoming the myelosuppresion of elevated chemotherapeutic agent doses (Kalifa et al., 1999; Linassier et al., 2001). A phase I study involving twenty children with resistant or recurrent brain tumours, investigated the efficacy of high dose cyclophosphamide and carboplatin with peripheral stem cell rescue. Six patients showed a complete response, two showed a partial response, four had stable disease and disease progression was seen in six cases (two could not be assessed). This treatment approach was tolerated and showed reasonable success (Foreman et al., 2005). Martinez et al. (2007) completed a retrospectively study over 7 years of 35 children with recurrent brain tumours who underwent high dose chemotherapy with autologous stem cell rescue. It was also concluded that this approach may be effective in specific histological groups, including high-grade astrocytoma and very young children.

Alternative therapies

Identification of molecular variations between normal brain and tumour cells has allowed therapeutic approaches to exploit these properties, specifically targeting replicative cells. Targeting therapies include inhibition of tumour growth through immunotherapy, inhibition of signal transduction pathways, anti-angiogenic therapy, and gene expression manipulation (Robertson, 2006).

Immunotherapeutic approaches include tumour vaccines or monoclonal antibodies for tumour-specific receptors that are linked to tumour toxins or radioisotopes. Dendritic antigen presenting cells sensitised to specific tumour antigens induce a cell-mediated immune response towards tumour cells. This has been shown to inhibit the overall growth of astrocytoma in adults and children (De et al., 2004; Yamanaka et al., 2005). Radiolabeled monoclonal antibodies targeting tumour growth factors, such as epidermal growth factor and its receptor, have been shown to induce tumour cell anti-proliferation activity *in vitro* and in animal models (Schechter et al., 2003). Phase I and II clinical trials in adults investigating these antibodies have produced promising results, showing disease stabilisation and prolonged survival (Boskovitz et al., 2004).

The inhibition of molecular pathways responsible for brain tumour growth, have also been investigated as a tumour treatment method. Growth factor receptors are linked to downstream molecular pathways involved in tumour cell proliferation and have been used as targets for monoclonal antibodies. The antibodies directed against specific receptors can inhibit signal transduction pathways inducing tumour cell apoptosis (Robertson, 2006). Recent monoclonal antibodies developed include, gefitinib (Iressa), which targets the epidermal growth factor receptor (EGFR) tyrosine kinase (Tremont-Lukats and Gilbert, 2003), imatinib (Gleevec), which targets the platelet derived growth factor-alpha receptor (PDGFRα) (MacDonald et al., 2001a) and erlotinib (Tarceva) targeting the ERBB2 receptor tyrosine kinase (Tremont-Lukats and Gilbert, 2003).

In addition, Rapamycin (Sirolimus) and a derivate (Everolimus) have been shown to inhibit the PTEN/PI3K/Ras/AKT/mTOR pathway in high-grade gliomas and are now undergoing phase II clinical trials in adult patients (Chang et al., 2005; Galanis et al., 2005). Furthermore, phase II clinical trials in paediatric and adult brain tumours are now being carried out for Tipifarnib (Zarnestra), a farnesyl transferase inhibitor which impairs proRas processing inhibiting the Ras signalling pathway (Tremont-Lukats and Gilbert, 2003; Cloughesy et al., 2006).

Angiogenesis is required for tumour growth. In brain tumours, several factors including α and β fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF) and angiogenin promote angiogenesis. These growth promoters are thought to be synthesised either by the tumour cells themselves, or cells in the tumour microenvironment such as macrophages or endothelial cells (Kerbel and Folkman, 2002; Robertson, 2006). Targets for anti-angiogenic agents include, endothelial cell proliferation, angiogenic factors or matrix metalloproteinases and vascular smooth muscle or integrin signalling (Puduvalli and Sawaya, 2000; Kerbel and Folkman, 2002). The cyclic peptide antagonist pentapeptide EMD 121974 (an integrin receptor antagonist) is currently in a phase I trial involving children with recurrent brain tumours. Preliminary results are promising with brain tumours showing responses to this agent (MacDonald et al., 2001b; Robertson, 2006).

Gene therapy is also being investigated as a treatment for adult and paediatric brain tumours. Prodrug activation (also known as "suicide therapy") delivers a gene to a

tumour cell which encodes a protein that sensitises the cell to a specific drug. Izquierdo et al. (1996) investigated the use of a modified herpes virus vector to treat malignant astrocytoma in 5 adult patients who had failed standard therapy and were expected to survive only a few weeks. The vector expressed the enzyme thymidine kinase which converts gangcyclovir to a toxic metabolite capable of killing tumour cells. Stereotactic intratumoural injection was used to administer the vector to the patient and this was followed by systemic gangcyclovir. Success was poor mainly due to transfection efficiency. However, this approach is now involved in phase I and II clinical trails in adults with recurrent high-grade GBMIV (Germano et al., 2003; Prados et al., 2003).

Tumour suppressor gene therapy is a similar approach in which mutated or deleted tumour suppressor genes are replaced with fully functional copies. *MMAC1/PTEN* is a tumour suppressor gene candidate commonly mutated in adult GBMIV. Following transduction of *MMAC1* into U87MG glioblastoma cells using adenovirus (MMCB), proliferation and anchorage-independent growth in soft agar was inhibited. *MMCB*-infected U87MG cells were also found to be non-tumorigenic in nude-mice. This suggests a strong *in vivo* tumour suppression activity of *MMAC1/PTEN* and that *in vivo,* gene transfer with this recombinant adenoviral vector has a potential use in cancer gene therapy (Cheney et al., 1998). More recently, infection with a combination of antisense-hTERT and wild-type-PTEN adenoviruses significantly inhibited human U251 GBMIV cell proliferation *in vitro* and glioma growth in a xenograft mouse model, providing further evidence that gene therapy could be a successful treatment approach for astrocytoma (You et al., 2007).

Anti-sense gene therapy inhibits the expression of genes involved in tumour development. An "anti-sense" gene is designed, which when synthesised, is a single-stranded RNA molecule complementary to the mRNA produced by the tumour target gene. If the gene is then expressed inside a tumour cell, the single-stranded RNA binds to the mRNA of the target gene, inhibiting ribosome apparatus function and preventing translation of the target gene. Urokinase plasminogen activator receptor (uPAR) and cathepsin B are over-expressed during astrocytoma progression. A study by Gondi et al. (2004) simultaneously down-regulated uPAR and cathepsin B in SNB19 glioma cell monolayers and spheroids, using an adenoviral vector carrying antisense uPAR and cathepsin B. The Ad-uAR-Cath B-infected cells showed a marked reduction in tumour

growth and invasiveness. These results indicate that this type of gene therapy could be used to inhibiting tumour growth, invasion, and angiogenesis.

Prognostic indicators in paediatric astrocytoma

Tumour histology

Tumour grade is the most important prognostic factor in paediatric astrocytoma, with a more malignant tumour being associated with reduced survival expectancy as previously discussed (Figure 1.7). Low tumour grade has been confirmed by several studies as a good prognostic indicator in children, with a 10-year survival expectancy of 80% or above(Lopez-Aguilar et al., 1997; Desai et al., 2001). Some sub-types of astrocytoma have been associated with a poorer prognosis. The presents of fibrillary histology has been shown to be a negative prognostic indicator in paediatric DAII (Smoots et al., 1998). Furthermore, poor prognosis has been associated with the gemistocytic DAII variant in adults but this is yet to be confirmed in paediatric astrocytoma (Watanabe et al., 1997b; Reis et al., 2001; Avninder et al., 2006; Martins et al., 2006; Geranmayeh et al., 2007).

Tumour location

Tumour location can impact paediatric patient prognosis and survival expectancy as only limited resection may be possible for tumours in difficult locations. Furthermore, variations in brain structure can allow rapid tumour infiltration promoting tumour growth and development (Robertson, 2006).

Total resection for PAI in the posteria fossa is possible in most cases and patients have a greater than 90% survival rate. Low-grade paediatric astrocytomas of the cerebral hemispheres often infiltrate surrounding areas and total resection is not always possible. When total resection is achieved, 10-year survival rates exceed 80% (Hirsch et al., 1989). Midline optic pathway or hypothalamic astrocytomas are rarely resectable as they often infiltrate critical structures. Further treatment for these paediatric tumours, including radiotherapy or chemotherapy, is dictated by other prognostic factors including, patient age and tumour histology. However, long term survival is approximately 75% (Petronio et al., 1991). Brain stem astrocytomas in children are extremely difficult to treat, as the location prevents surgical intervention and patients have a median survival of less than a year (Hargrave et al., 2006). High-grade paediatric

supratentorial astrocytomas are initially treated with surgery and maximum tumour resection is associated with prolonged survival (Wisoff et al., 1998). However, the 2-year survival rate of paediatric patients with high-grade tumours in this location is only approximately 20% (Broniscer and Gajjar, 2004).

Age

Young infants diagnosed with an astrocytoma have a worse prognosis compared to older children diagnosed with a similar tumour. Studies investigating the difference in prognosis depending on patient age have grouped patients as below 3, 2 or 1 years of age compared to older children (Raimondi and Tomita, 1983; Duffner et al., 1986; Ambrosino et al., 1988; Asai et al., 1989; Haddad et al., 1991; Duffner et al., 1993; Cohen et al., 1993; Jovani et al., 1998; Duffner et al., 1999; Sala et al., 1999; Rivera-Luna et al., 2003; Rivera-Luna et al., 2007). Moreover, Desai et al. (2001) investigated prognostic factors for cerebellar astrocytoma in 102 children and found that patient age did not affect outcome. However, the youngest patient in the study was 10 months old, consequently the younger age group was not sufficiently represented in this study. As previously discussed, neurotoxicity from RT is more frequent and severe in very young children, in some cases contributing to poor prognosis and quality of life (Jenkin et al., 1998).

Molecular genetic markers

Predictive genetic markers in paediatric astrocytoma are limited. However, a number of studies have investigated the association between copy number alterations (CNAs) and outcome. Reduced survival was associated with gain of 1q in 30% of paediatric AAIII and 54% of paediatric GBMIV, $p < 0.001$ and $p < 0.05$ respectively (Rickert et al., 2001). A study of CNAs in low-grade paediatric astrocytoma suggested that patients with tumours that had aberrations survived longer, although this was not significant (Orr et al., 2002). Furthermore, loss of 17p has also been associated with reduced survival in paediatric astrocytoma (Cogen, 1991)

Mutations or over-expression of *TP53* in paediatric astrocytoma has been associated with reduced patient survival (Drach et al., 1996; Pollack et al., 2002; Ganigi et al., 2005). Pollack et al. (2002) found that there was a significant association between TP53 over-expression and shorter progression-free survival (PFS) and overall survival (OS).

PFS and OS were 5.5 months and 14 months respectively, compared to 25 months and >4 years for those patients with tumours without TP53 over-expression. In addition, there was a significant association between *TP53* mutation, reduced PFS and shorter OS. Median PFS and OS for patients with *TP53*-mutated tumours were 6 months and 16 months respectively, versus 16 months and 25 months for those patients with tumours that did not have *TP53* mutations (Pollack et al., 1997). A further study found a significant association between increased TP53 immunopositive tumour cells and reduced survival in paediatric GBMIV (Drach et al., 1996). Moreover, an investigation of TP53 immunoreactivity in DAII and AAIII demonstrated a link between increased TP53 staining in AAIII and reduced survival, but this was not found to be significant (Ganigi et al., 2005).

Increased EGFR and bcl-2 immunoreactivity was observed in 25.9% and 33.3% of paediatric GBMIV respectively, but this was not significantly associated with survival. However, combined increased TP53 and bcl-2 immunoreactivity was associated with reduced survival (Ganigi et al., 2005). Moreover, *PTEN* deletions have also been associated with poor prognosis in paediatric AAIII and GBMIV (Raffel et al., 1999).

The methylation status of *MGMT* is a good predictive marker of response to alkylating agents (Esteller et al., 2000a; Soejima et al., 2005). The relationship between *MGMT* methylation status, chemotherapy response and patient survival has been studied in paediatric GBMIV patients. A randomized trial comparing radiotherapy alone and radiotherapy combined with concomitant or adjuvant treatment with temozolomide, demonstrated that *MGMT* promoter methylation conferred a survival benefit to paediatric GBMIV patients treated with temozolomide and radiotherapy. However, a strong association was also seen between *MGMT* promoter methylation and overall survival regardless of treatment (Donson et al., 2007).

Genetics and chromosomal alterations of astrocytoma

The study of paediatric astrocytoma is hindered by several factors, the first being the rarity of this tumour, especially high-grade tumours. Secondly, the location of many paediatric astrocytoma prevents resection, limiting the amount of tissue available for study. Furthermore, patient consent is given in fewer cases for the study of paediatric astrocytoma compared to adults. This is an understandable parental choice. Moreover,

in the experience of our laboratory and collaborators, it is often difficult to generate good chromosome metaphase spreads for tumour cytogenetic analysis. Paediatric astrocytoma often have a low mitotic index and reduced doubling time compared to adults, decreasing the number of mitoses for metaphase spread preparation. This has hindered genetic investigation of paediatric astrocytoma compared to that of their adult counterparts.

Genetics and chromosome alterations in adult astrocytoma

Phenotypic characteristics of all tumour cells include increased proliferation, increased invasion, angiogenic properties and evasion of apoptosis. A number of molecular mechanisms have been identified in cellular transformation and malignancy that can be linked to these characteristics including 1) inactivation of Rb and TP53 cellular pathways; 2) activation of Ras and/or other growth promoting pathways; 3) evasion of apoptosis; 4) telomerase activation or alternative mechanisms of cellular immortalisation; 5) angiogenic activity (Bocchetta and Carbone, 2004).

Oncogenes are generally mutated forms of normal cellular genes (proto-oncogenes), which usually influence cell proliferation. They are genes that code for growth factor receptors, proteins involved in signal transduction or transcription factors (Bocchetta and Carbone, 2004). Mutations in proto-oncogenes can give rise to cellular genes that are constitutively active and behave like viral oncogenes. For example, substitution of the glycine at codon 12 of the *K-ras* proto-gene produces a constitutively active oncogenic *ras* allele, that is a marker in pancreatic carcinoma (Daus et al., 1995). Failure of cellular mechanisms to respond to this differential expression promotes tumour formation.

Genes that can promote tumour development through loss of function are termed tumour suppressor genes. These can be transcription factors such as TP53, proteins that interact with other proteins to inhibit kinase activity involved in cell cycle progression such as CDKN2A and CDKN1A, or proteins that affect cellular localisation of other proteins (Malumbres and Barbacid, 2001).

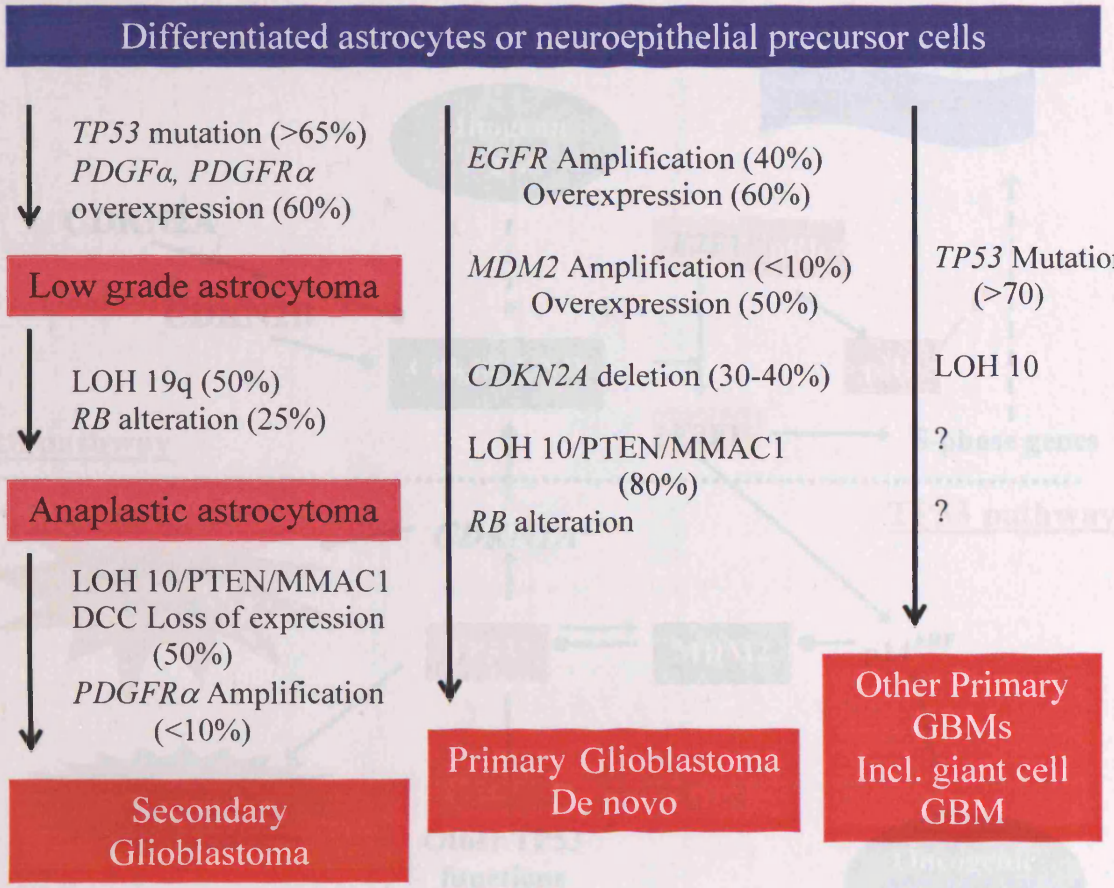
Rb and TP53 pathways

The cell cycle can be arrested by tumour suppressor genes of the TP53 and Rb pathways that are activated by aberrant proliferative stimuli or DNA damage. After cell arrest, there is an opportunity to rectify DNA damage where necessary. If this is not possible or damage is irreparable, the cell enters senescence or undergoes apoptosis, preventing replication of the damaged genome (Bocchetta and Carbone, 2004). The role of the TP53 and Rb pathways is to maintain low levels of inactive TP53 in the absence of genomic damage allowing normal cell cycle function (Rathore et al., 1999; Kohn, 1999; Besson and Yong, 2001; Collins, 2002; Mirza et al., 2003). The majority of tumours have mutations in multiple tumour suppressor genes causing major disruptions to the TP53 and Rb pathways (Hahn and Weinberg, 2002). The basic role of TP53 in astrocytoma is discussed here, although the known multiple gene interactions have not been included. Current detailed information regarding this gene can be found at http://www-p53.iarc.fr/p53resources.html.

Primary and secondary adult GBMIV have different genetic profiles and chromosomal alterations (Figure 1.8). However, it is apparent that the TP53 and Rb pathways are disrupted in both through different mechanisms. Figures 1.8 and 1.9 illustrate genomic aberrations and differential gene expression identified in primary and secondary adult GBMIV (Konopka and Bonni, 2003; Ichimura et al., 2004). Furthermore, these two distinct pathways are now recognised by the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. This database is a collection of manually drawn pathway maps representing current knowledge of molecular interactions and reaction networks (Kanehisa and Goto, 2000) (Figures 1.10 and 1.11).

The activation of TP53 transactivates many genes with varied functions. The transcription of *CDKN1A* is activated by TP53 and inhibits the Rb pathway. *MDM2* transcription is also initiated by TP53, which itself acts as a negative regulator of TP53. Cell cycle progression is also regulated through the release of E2F transcription factors by Rb phosphorylation, which initiates $p14^{ARF}$ transcription. This protein inhibits MDM2 preventing TP53 degradation, consequently increasing TP53 cellular concentration and inducing cell cycle arrest or apoptosis (Rathore et al., 1999; Kohn, 1999; Besson and Yong, 2001; Collins, 2002; Mirza et al., 2003).

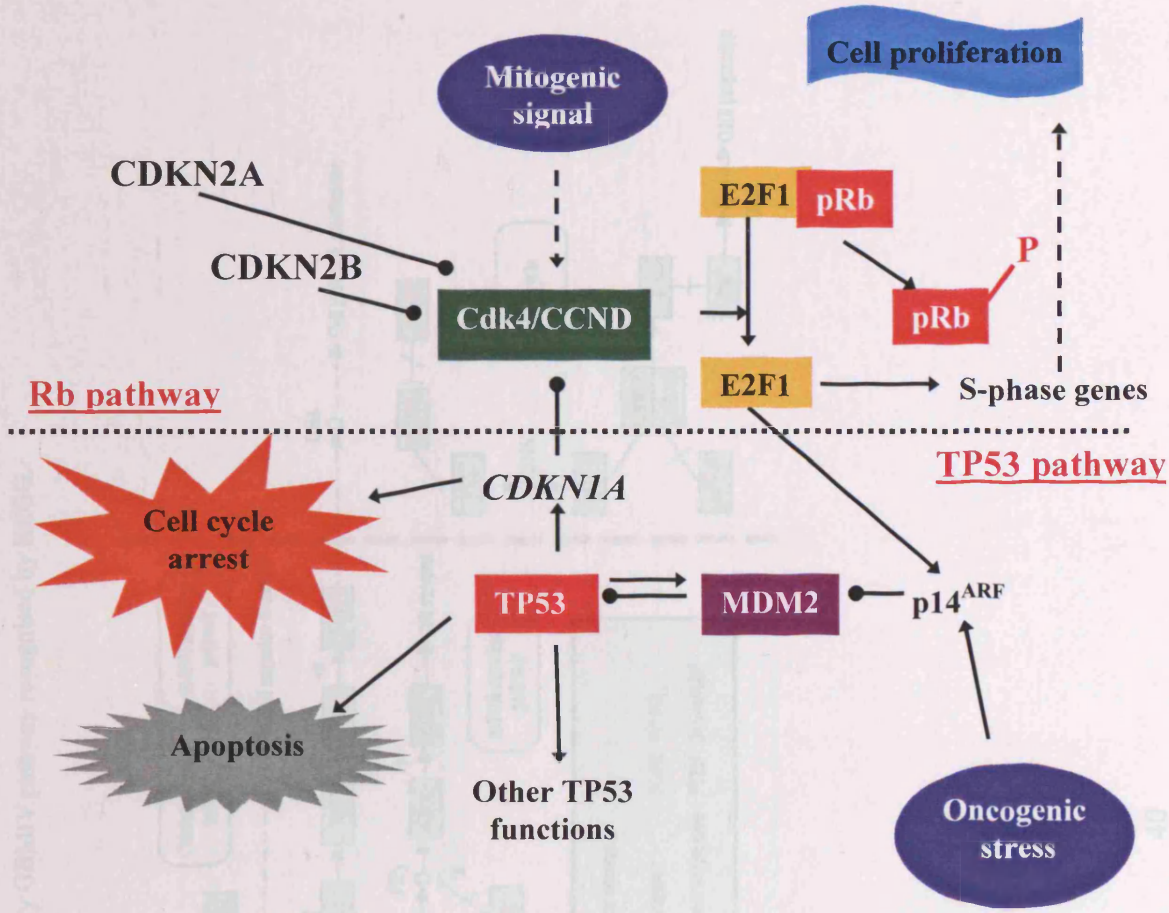
Figure 1.8 The genetic differences between primary and secondary adult glioblastoma.



Modified from Pathology and Genetics Tumours of the Nervous System (Louis et al., 2007a).

The Rb pathway controls cell cycle progression from G1 to S phase. The release of E2F transcription factors through pRb phosphorylation initiates the transcription of S phase genes. Mitogenic stimuli up-regulates CCND1, CCND2 and CCND3, which bind CDK4 or 6. This complex phosphorylates the Rb protein, causing the release of E2F transcription factors. Phosphorylated Rb also modulates chromatin structure (Zhang and Dean, 2001). CDK inhibitors, CDKN2B and CDKN2A,compete with the cyclins to bind CDK4 or 6, blocking complex formation and the Rb pathway (Rathore et al., 1999; Kohn, 1999; Besson and Yong, 2001; Collins, 2002). The loss or mutation of *TP53* is found in 60% of adult DAII, AAIII and secondary GBMIV (Frankel et al., 1992; Rasheed et al., 1994; Nozaki et al., 1999; Ichimura et al., 2000). This promotes cell proliferation, as does the over-expression or amplification of *MDM2*. *MDM2* is located on chromosome 12q13-15, a common region of gain in these tumours (Reifenberger et al., 1996; Ichimura et al., 2000; Ranuncolo et al., 2004).

Figure 1.9 TP53 and Rb1 cell cycle pathways



The chromosomal region 9p21 is homozygously deleted in 30-40% of adult primary GBMIV and 10% of AAIII, but not in DAII. This region contains the *CDKN2A* locus encoding both *CDKN2A* and $p14^{ARF}$. The deletion of *CDKN2A*, promotes cell proliferation through the disruption of the Rb and TP53 pathways (Jen et al., 1994; Ueki et al., 1996; Ichimura et al., 2000; Ranuncolo et al., 2004). Hypermethylation of the promoter region of these genes, has also been found in astrocytoma (Fueyo et al., 1996; Nakamura et al., 2001a; Gonzalez-Gomez et al., 2003a). Furthermore, *Rb* loss or promoter hypermethylation has been found in approximately 25% of astrocytoma (Ueki et al., 1996; Nakamura et al., 2001b).

Figure 1.10 Adult primary GBMIV pathway recognised by KEGG.

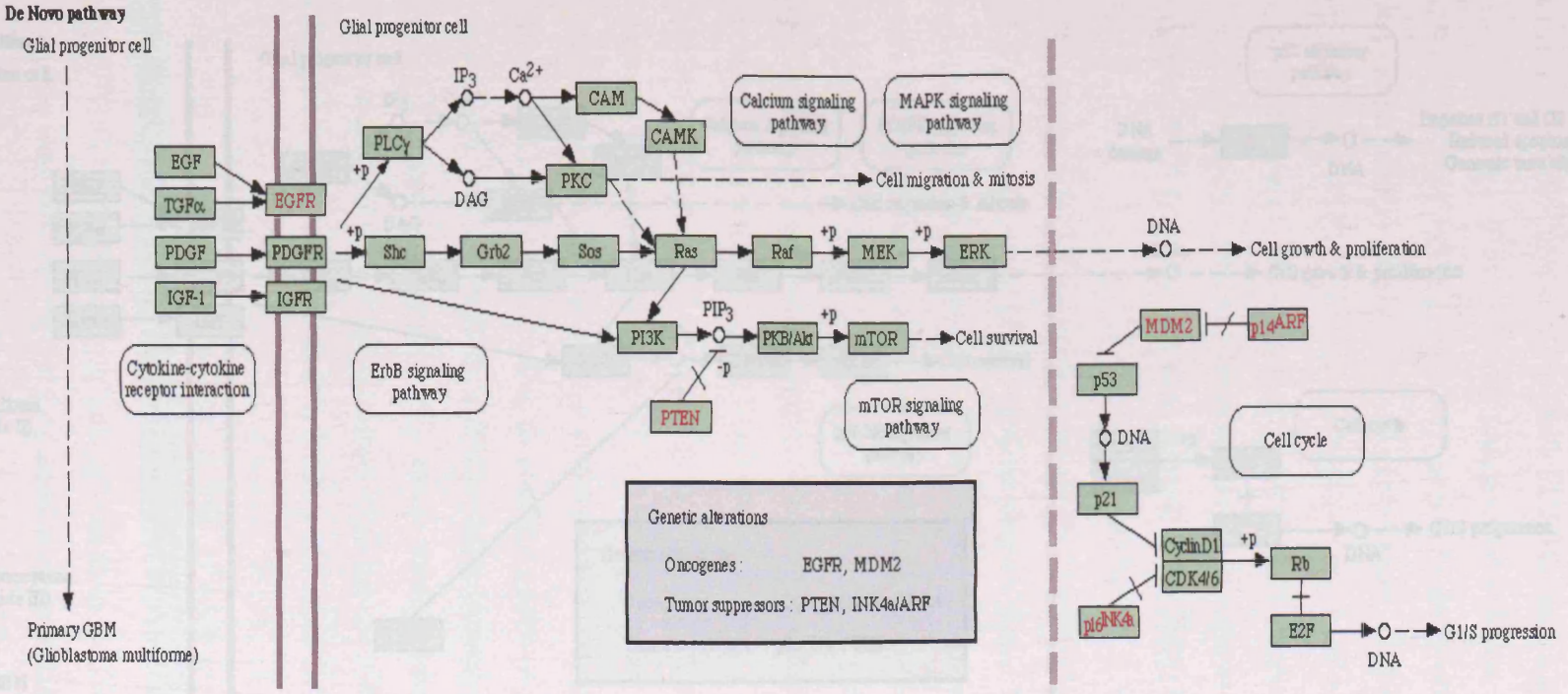
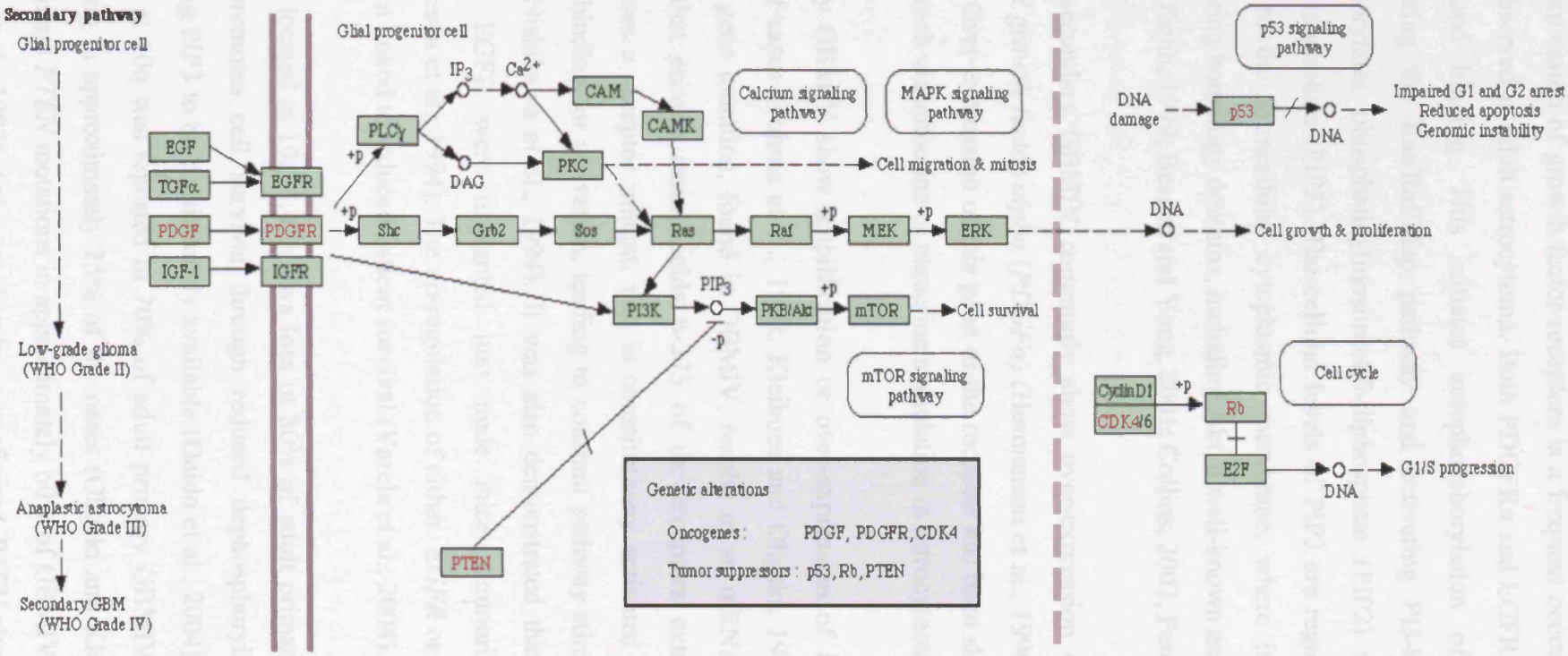


Figure 1.11 Adult secondary GBMIV pathway recognised by KEGG.



Activation of Ras and growth promoting pathways

Over-expression of growth factor receptors is a frequent occurrence in cancer and has been observed in adult astrocytoma. Both PDGFRα and EGFR dimerise once activated by ligand binding. This initiates autophosphorylation of cytoplasmic domains stimulating the Ras/Raf/Map pathway and activating PI3-kinase. PI3-kinase then phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The cellular levels of PIP3 are regulated by PTEN. PIP3 is bound to the intracellular cytoplasmic membrane, where it recruits proteins with pleckstring homology domains, including Akt, a well-known anti-apoptotic and survival factor (Kohn, 1999; Besson and Yong, 2001; Collins, 2002; Pandita et al., 2004).

Adult secondary GBMIV commonly show over-expression of *PDGFRα* or *platelet derived growth factor alpha* (*PDGFα*) (Hermanson et al., 1996; Kleihues and Ohgaki, 1999). Over-expression of this gene or the receptor has been shown to promote tumour self growth via autocrine or paracrine stimulation in astrocytoma (Lokker et al., 2002).

Primary GBMIV show amplification or over-expression of *EGFR* in approximately 50% of cases (Tohma et al., 1998; Kleihues and Ohgaki, 1999). The most common *EGFR* gene mutation, found in GBMIV, results in an mRNA molecule lacking 801 bases that encode amino acids 6-273 of the receptors extracellular domain. This transposes a receptor mutant, that is constitutively activated and no longer requires ligand binding for activation, leading to constant pathway stimulation (Ekstrand et al., 1994; Nishikawa et al., 1994). It was also demonstrated that when cells expressing mutant EGFR were implanted into nude mice, tumourigenesis was increased (Nishikawa et al., 1994). The up-regulation of either *EGFR* or *PDGFRα* in some DAII has been linked to reduced patient survival (Varela et al., 2004).

PTEN, located at 10q23.4, shows loss in 80% of adult primary GBMIV. Loss of this gene promotes cell survival through reduced dephosphorylation of PIP3 to PIP2 allowing PIP3 to be continuously available (Daido et al., 2004). Loss of heterozygosity (LOH) at 10q was reported in 70% of adult primary GBMIV, with *PTEN* mutations occurring in approximately 25% of the cases (Ohgaki and Kleihues, 2005). A further study found *PTEN* mutations in approximately 60% of GBMIV also showing 10q LOH (Wang et al., 1997). Other studies have confirmed *PTEN* alterations in adult primary

GBMIV and associated the disruption of this gene with reduced patient survival (Kato et al., 2000; Krex et al., 2003; Roversi et al., 2006; Homma et al., 2006).

Cell immortalisation

Cell immortalisation is a further event that contributes to malignant growth of astrocytoma. In most cases, this is thought to be achieved through the maintenance of chromosome telomeres. These regions are 5-15Kbp in length and consist of 5'-TTAGGG-'3 tandem repeats at the end of each chromosome. The 3' overhang of the repeat is arranged in a T-loop structure that protects the individual chromosome ends from end to end chromosome fusion and prevents the single strand being recognised as a double strand break, triggering the cell DNA repair system (Griffith et al., 1999). Under normal cell division and chromosome replication, the telomere region shortens because DNA polymerase uses RNA primers that are degraded after elongation (Moyzis et al., 1988). Once a telomere has shortened beyond the critical threshold, the DNA ends become structurally unstable. This leads to TP53 activation resulting in either replicative senescence or crisis. Deletion of TP53 significantly attenuates cellular effects of telomere dysfunction, but only during the earliest stages of genetic crisis. (Chin et al., 1999).

Germinal cells and tumour cells evade this mechanism that restricts cellular replication, through the activation of telomerase. This enzyme is a ribo-protein complex that adds TTAGGG repeats to the telomeres preventing shortening (Bocchetta and Carbone, 2004). It has been suggested that over 85% of human cancers have measurable levels of telomerase activity allowing indefinite proliferation (Shay and Bacchetti, 1997). Tumours without telomerase activity are thought to maintain telomere length through a process known as alternative lengthening of telomeres (ALT). This involves nonreciprocal recombination between telomeres of different chromosomes (Bryan et al., 1997). Telomerase activity has been found in both primary and secondary adult GBMIV, and associated with increased *TP53* mutation (Harada et al., 2000). Furthermore, a study by Chen et al. (2006) found an association between *TP53* mutation and the ALT mechanism and between wild-type *TP53* and telomerase activity, suggesting that regardless of *TP53* mutation status, a mechanism promoting telomere maintenance is active.

Angiogenesis

Angiogenesis is the formation of new blood vessels from those that already exist and is controlled through a balance of pro-angiogenic and anti-angiogenic factors. High levels of angiogenesis occur during embryonic development compared to that found in adults where the process is limited to the menstrual cycle and tissue repair and remodelling. This complex physiological process has been investigated in astrocytoma since the early 1970's and is now recognised as an essential mechanism involved in tumour development and progression (Folkman, 1971; Brem, 1976). Moreover, during tumour development the angiogenic signal balance is pushed towards new vessel growth to create a blood vessel network that facilitates nutrient and waste product exchange and allows tumour growth to exceed 1-2mm^3 (Folkman, 1990). Tumour cells that are more than ten cell layers from a vessel are hypoxic and it is this lack of oxygen that inhibits tumour growth (Evans et al., 2001). Low-grade astrocytoma are 'non-angiogenic tumours' as they utilise normal brain vasculature as a blood supply. Furthermore, the extent of tumour vascularity correlates with increasing tumour malignancy and adult GBMIV have been shown to be the most vascularised astrocytoma (Korkolopoulou et al., 2002). These tumours also show areas of necrosis linked to extensive tissue hypoxia. Moreover, WHO classification grading criteria for astrocytoma includes microvascular proliferation and necrosis (Louis et al., 2007a).

In order to quantify angiogenesis in astrocytoma, specific vessel parameters have been investigated including microvessel density and microvessel size and shape according to branching (Folkerth, 2000; Sharma et al., 2006). Increased microvessel density and branching has been associated with poor prognosis in adult astrocytoma (Leon et al., 1996; Abdulrauf et al., 1998). Furthermore, a sub-group of low-grade adult astrocytoma with shorter patient survival showed increased numbers of microvessels at diagnosis, suggesting that a number of low-grade adult astrocytoma may have increased malignant potential (Abdulrauf et al., 1998). In a small group of six paediatric GBMIV, vascular hyperplasia was also associated with reduced patient survival (Germano et al., 2001). Moreover, "sprouting" of new capillaries and an immature vascular system was linked to paediatric AAIII compared to PAI and proposed as a rationale for anti-angiogenic treatment approaches in this patient group (Gesundheit et al., 2003).

A plethora of growth factors, cytokines and respective receptors are involved in

angiogenesis causing basement membrane degradation, endothelial cell migration, proliferation and tubular formation (Folkman, 1992). These include, VEGF, FGF, PDGF, EGF, TGFα, TGFβ, HGF, CYR61, CTGF, IGF-1, IL-6, IL-8, TNFα, angiopoietins and ephrins. The most prominent factor is VEGFα, an endothelial cell mitogen that binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 and initiates endothelial cell proliferation, migration and development of new blood vessels (Kanno et al., 2000; Yancopoulos et al., 2000). Moreover, the expression of this factor is mediated by hypoxia-inducible factor-1 (HIF-1), a factor stabilised under hypoxic conditions (Forsythe et al., 1996). *VEGF* expression was found in adult astrocytoma cells but markedly increased in GBMIV cells. In the same study, the expression of the *VEGF-1 receptor* was shown to be up-regulated in tumour endothelial cells compared to normal brain epithelium (Plate et al., 1992). The importance of the VEGF-1 receptor in vasculogenesis has since been confirmed (Fong et al., 1995; Shalaby et al., 1995) Furthermore, the expression of *VEGF* is affect by the PI3K signalling pathway under both hypoxic and normoxic conditions and by the MAPK/ERK pathway during normoxia (Jiang et al., 2000; Woods et al., 2002). This suggests that genetic mutations common to adult GBMIV may influence *VEGF* expression. The loss or mutation of *PTEN* frequently found in primary GBMIV activates the PI3K signalling pathway, inadvertently up-regulating *VEGF* expression and promoting angiogenesis. Moreover, the *VEGF* promoter region contains transcription factor binding sites for SP1, a target of wild-type TP53. Loss or mutation of *TP53* in secondary GBMIV may promote *VEGF* transcription via SP1 activity (Zhang et al., 2000).

Angiopoietins are recognised as partners of VEGF in angiogenesis. Angiopoietin-1 (Ang-1) and its tyrosine kinase receptor, Tie-2, play a complementary role to VEGF and the VEGF-1 receptor by ensuring vascular stability and correct vessel formation (Suri et al., 1996; Zadeh et al., 2004). Furthermore, transgenic mice over-expressing *VEGF* and *Ang-1* show hypervascularity with an increase in both vessel number and size (Thurston et al., 1999). Ang-2 has been proposed as both an agonist and antagonist, inhibiting the actions of Ang-1 and the Tie-2 receptor but promoting angiogenesis, compared to Ang-1 that promotes vascular stability. It is thought that Ang-2 inhibits this action, returning already mature vessels of the brain to a plastic, destabilised state, sensitive to angiogenesis and further vessel formation (Holash et al., 1999).

In adult GBMIV, increased immunohistochemical staining of Tie-2 was found in tumour endothelium compared to normal human brain. Ang-1 staining was shown in all tumour cells compared to Ang-2, which was restricted to a small number of GBMIV blood vessel endothelial cells. Immature capillaries with few periendothelial support cells showed increased Ang-2 staining, compared to larger vessels with many periendothelial support cells that showed reduced staining (Stratmann et al., 1998).

Angiogenesis is a complex process involving a large number of growth factors only named here. All levels of tissue and cellular organisation are involved, as well as secreted ligands, receptors, ECM proteins, transcription factors and intracellular signalling molecules. Moreover, tumour microenvironment including, tissue oxygen level also plays a significant role (Kargiotis et al., 2006). Furthermore, common genetic alterations that occur in adult astrocytoma and to a greater extent in GBMIV, promote angiogenesis allowing tumour growth and infiltration (Kaur et al., 2004).

Copy number alterations

Genomic alterations involving chromosome copy number gains and losses impact differential gene expression and adult astrocytoma development. Many altered regions are thought to be the location of novel candidate tumour suppressor genes and oncogenes. Although PAI are most common in children, they do occur in adults. The majority of PAI found in adults show normal karyotypes, as reported by Sanoudou et al (2000a). In this study normal karyotypes were identified in 5 of 7 adult PAI, with the remaining 2 tumours exhibiting multiple complex aberrations.

The most common chromosomal alterations found in adult DAII include gain of part or all of chromosome 7, an increase in 8q copy number (whole arm or specific regions) and loss of a single sex chromosome (Schrock et al., 1996; Perry et al., 1997; Nishizaki et al., 1998; Hirose et al., 2003). Other regions of chromosome or arm alterations reported in this tumour include -1p, -4q, +5p, +9, +12, -19q, +19p (Schrock et al., 1996; Nishizaki et al., 1998). Furthermore, loss of chromosome 10 or regions on 6q are uncommon but have been identified (Perry et al., 1997; Nishizaki et al., 1998; Miyakawa et al., 2000). Allelic loss of 17p has also been detected in adult DAII and correlates with *TP53* mutations in the same tumour (von Deimling et al., 1992).

In adult AAIII, gain of part or all of chromosome 7 is also the most common chromosome alteration, as seen in adult DAII (Nishizaki et al., 1998; Kunwar et al., 2001; Koschny et al., 2002). The loss of 17p, Xq and regions on 6q have also been found in this tumour (von Deimling et al., 1992; Miyakawa et al., 2000). Additional chromosome gains and losses reported in adult AAIII include -1p, -4q, -8q, +10p, -11q, -11p, -12q and -13q and -9p, -10q, -13q and + 19q, more common in adult GBMIV (Nishizaki et al., 1998; Kunwar et al., 2001). Furthermore, alterations involving -4q, +7p, -10 and +19 were more common in patients above 45 years of age, compared to -11p, which was more common in patients below 45 years of age (Kunwar et al., 2001).

The most common chromosome alterations found in adult GBMIV include the loss of chromosomes 9p, 10, 13, 14, 17p and 22q and gain of chromosomes 7, 19 and 20. Overall, chromosomal loss is more common than gain (Schrock et al., 1994; Mohapatra et al., 1998; Harada et al., 1998; Nishizaki et al., 1998; Koschny et al., 2002). Multiple regions of deletion on 6q have also been reported in this tumour (Mohapatra et al., 1998; Miyakawa et al., 2000). Furthermore, defined regions frequently altered in adult GBMIV include +7p13-p12, +7p31, +8q24.1-q24.2, -9p21, -10p12-p11, -10q22-qter, -13q21-13q22 and 20q13.1-13.2 (Harada et al., 1998; Nishizaki et al., 1998).

Many of these regions contain genes known to play a role in adult astrocytoma development or tumour progression including *GAC1* at 1q32, *PDGFRα* at 4q12, *PDGFRβ* at 5q34, *EGFR* at 7p12, *CDK6* and *MET* at 7q21-7q31, *C-MYC* at 8q24.1, *PBX3*, *VAV2* and *ABL* at 9q34, *PTEN* at 10q23.3, *MXI-1* at 10q25-q26, *CCD1* at 11q13, *CDK4* and *MDM2* at 12q13-q15, *Rb* at 13q14.2, *TP53* at 17p13, *DCC* at 18q21-q22 and *ERCC1*, *ERCCC2* and *BAX* at 19q13 (Koschny et al., 2002). A less documented region at 3q25.3 contains the *VHL* gene reported to be lost in 40% of adult astrocytoma (Kanno et al., 1997). The 1p arm has also been studied in high-grade adult tumours with 20% of cases showing loss (Smith et al., 1999). Moreover, the *TP73* gene, homologous to *TP53,* is located in this region. *TP73* promoter hypermethylation has been found in some adult GBMIV as an alternative mechanism for gene expression loss (Watanabe et al., 2002).

The deletion of chromosome 10 is the most common aberration found in adult GBMIV, occurring in 80% of cases. The tumour suppressor gene, *PTEN,* was identified at 10q23 and is most frequently lost in these tumours. Furthermore, DMBT1, located at 10q25-26

the region most frequently deleted on chromosome 10, was also shown to be lost in 50% of tumours (Suzuki et al., 2004). Other genes have been suggested as tumour suppressors lost due to 10q deletions including, *MXI1* and *LGI1* (Wechsler et al., 1997; Mollenhauer et al., 1997; Fujisawa et al., 1999; Chernova et al., 2001; Wessels et al., 2004).

Epigenetics

In normal cells, epigenetics can be defined as the mechanisms that preserve inheritable gene expression patterns and functions without altering the genome sequence. The epigenetic network is complex but can be summarised into four layers; DNA methylation, chromatin remodelling, histone modification, and non-coding RNAs. In tumours and cancer it is evident that major disruptions often occur in the epigenetic network (Esteller, 2006). Moreover, promoter hypermethylation is the most common epigenetic alteration in tumours causing loss or down-regulation of tumour suppressor gene expression. Hypermethylation describes an increase in the number of methylated CpG dinucleotides in a CpG island compared to normal levels.

The methylation of cytosine residues located within the dinucleotide CpG do not occur randomly within the genome, but cluster in CpG rich regions known as CpG islands that are primarily located at the 5'end of genes. These islands often span the promoter, untranslated region and exon 1, indicating the beginning of a gene sequence. In the DNA of normal cells, 5-methylcytosine (5mC) constitutes 0.75%-1% of all nucleic acid bases and 3-4% of methylated cytosines (Paz et al., 2002). Furthermore, in normal cells, methylation controls the gene expression of imprinted genes, X-chromosome genes in the female, germ line specific genes and tissue-specific genes (Feinberg et al., 2002). In comparison, CpG dinucleotides are often unmethylated in normal cells and hypermethylated in tumour cells, silencing gene expression (Esteller, 2005).

Hypomethylation has also been documented in tumour cells and has been attributed to tumourigenesis. In such cases, tumour cells have been found to have 20% to 60% less genomic 5mC than normal cells, occurring mainly at coding regions and introns, compared to hypermethylation, that is usually found at promoter regions. Reduced levels of 5mC are thought to invoke chromosome instability, reactivation of transposable elements and loss of imprinting, promoting mitotic recombination and loss

of heterozygosity (Esteller, 2005).

Genes that control global genome methylation have been investigated in many tumours and cancer. A family of genes with DNA methyltransferase activity including DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, are thought to play a role in the maintenance of promoter region methylation. Differential expression of only DNMT1 in colon cancer cells was shown to have little effect on global methylation. However, when both DNMT1 and DNMT3a were down-regulated, methyltransferase activity was lost and methylation was reduced to less than 5% of the original level (Rhee et al., 2002).

Tumour suppressor genes are frequently hypermethylated in tumours with significant consequences that provide cells with a selective advantage (Table 1.2). The cell cycle inhibitor *CDKN1A* is hypermethylated in a variety of cancers allowing cells to proliferate and escape senescence (Merlo et al., 1995). Other genes involved in tumour cell cycle control found to be hypermethylated include the *Rb* gene and *CDKN2B*. Regulation of *Rb* gene methylation status has also been linked to chromatin conformation at the promoter region, suggesting that several epigenetic mechanisms are simultaneously involved (De La Rosa-Velazquez IA et al., 2007). Several genes in the TP53 network including *CDKN2A* and *TP73* have been found to be hypermethylated. Methylation, and consequent silencing of *CDKN2A*, allows uninhibited MDM2 to induce TP53 degradation (Esteller et al., 2001). Furthermore, genes of the APC/β-catenin, E-cadherin and Wnt signalling pathways are often methylated in tumours including *APC*, *E-cadherin*, *H-cadherin*, *FAT* and *SFRP1* (Graff et al., 1995; Esteller et al., 2000b; Toyooka et al., 2001; Paz et al., 2003).

Alterations in methylation patterns also affect DNA repair mechanisms in tumours. Silencing of the mismatch DNA repair gene *hMLH1*, has been found in colorectal, endometrial and gastric tumours in conjunction with microsatellite instability (Herman et al., 1998; Esteller et al., 1999; Fleisher et al., 1999). The methylation of this gene in adult GBMIV has also been linked to a positive response in patients given nitrosoureas as part of a chemotherapy regime (Fukushima et al., 2005). Furthermore, the methylation status of *MGMT* is a good predictive marker of response to alkylating agents as previously discussed (Esteller et al., 2000a; Soejima et al., 2005; Hegi et al.,

2005) The same relationship has recently been documented in paediatric GBMIV patients, but a strong association was also seen between *MGMT* promoter methylation and overall survival, regardless of treatment (Donson et al., 2007).

Table 1.2 Genes hypermethylated in specific tumours and cancers and the molecular consequences.

Gene	Function	Location	Tumour Profile	Consequences
CDKN2A	Cyclin-dependent kinase inhibitor	9q21	Multiple types	Entrance in cell cycle
$p14^{ARF}$	MDM2 inhibitor	9q21	Colon, stomach, kidney	Degradation of p53
CDKN2B	Cyclin-dependent kinase inhibitor	9q21	Leukaemia	Entrance in cell cycle
hMLH1	DNA mismatch repair	3p21.3	Colon, endometrial, stomach, glioblastoma	Frameshift mutations
MGMT	DNA repair of 06-alkyl-guanine	10q26	Multiple types	Mutations, chemosensitivity
GSTP1	Conjugation to glutathione	11q13	Prostate, breast, kidney	Adduct accumulation
BRCA1	DNA repair, transcription	17q21	Breast, ovary	Double strand breaks
p73	P53 homolog	1p36	Lymphoma	Unknown
LKB1/STK11	Serine/threonine kinase	19p13.3	Colon, breast, lung	Unknown
ER	Estrogen receptor	6q25.1	Breast	Hormone sensitivity
PR	Progesterone receptor	11q22	Breast	Hormone sensitivity
AR	Androgen receptor	Xq11	Prostate	Hormone sensitivity
PRLR	Prolactin receptor	5p13-p12	Breast	Hormone sensitivity
RARβ2	Retinoic acid receptor	3p24	Colon, lung, head and neck	Vitamin sensitivity
RASSF1A	Ras effector homolog	3p21.3	Multiple types	Unknown
NORE1A	Ras effector homolog	1q32	Lung	Unknown
VHL	Ubiquitin ligase component	3p25	Kidney, hemangioblastoma	Loss of hypoxic response
Rb	Cell cycle inhibitor	13q14	Retinoblastoma	Entrance in cell cycle
THBS-1	Thrombospondin-1 antiangiogenic	15q15	Glioma	Neovascularisation
CDH1	E-cadherin, cell adhesion	16q22.1	Breast, stomach, leukaemia	Dissemination
CDH13	H-cadherin, cell adhesion	16q24	Breast, lung	Dissemination
FAT	Cadherin, tumour suppressor	4q34-35	Colon	Dissemination
HIC-1	Transcription factor	17p13.33	Multiple types	Unknown
APC	Inhibitor of β-catenin	5q21	Aerodigestive tract	Activation of β-catenin route
SFRP1	Secreted frizzled-related protein-1	8p12-p11	Colon	Activation WNT signalling
COX-2	Cycooxygenase-2	1p25	Colon, stomach	Anti-inflammatory resistance
SOCS-1	Inhibitor of JAK/STAT pathway	16p13.13	Liver, myeloma	JAK2 activation
SOCS-3	Inhibitor of JAK/STAT pathway	17q25	Lung	JAK2 activation
GATA-4	Transcription factor	8p23-p22	Colon, stomach	Silencing of target genes
GATA-5	Transcription factor	20q13	Colon, stomach	Silencing of target genes
SRBC	BRAC-1 binding	1p15	Breast, lung	Unknown
SYK	Tyrosine kinase	9q22	Breast	Unknown
RIZ1	Histone/protein methyltransferase	1p36	Breast, liver	Aberrant gene expression
DAPK	Pro-apoptotic	9q34.1	Lymphoma, lung, colon, glioblastoma	Resistance to apoptosis
TMS	Pro-apoptotic	16p11	Breast, glioblastoma	Resistance to apoptosis
TPEF/HPP1	Transmembrane protein	2q33	Colon, bladder	Unknown

Taken from Manel Esteller 2005 (Esteller, 2005).

Few studies have investigated methylation in paediatric astrocytoma. In adult GBMIV, methylation of *DAPK* and *TMS1* has been found at low frequencies and *THBS1* methylation has been shown in both low and high-grade astrocytoma (Li et al., 1999; Gonzalez-Gomez et al., 2003a). A detailed investigation of the methylation status of 34 genes in 54 adult astrocytomas of various grades identified methylation of *RASSF1A*, *TP73*, *AR*, *MGMT*, *CDH1*, *OCT6*, *MT1A*, *WT1*, and *IRF7* that were methylated in 69.8%, 47.2%, 41.5%, 35.8%, 32%, 30.2%, 30.2%, 30.2% and 26.4% of cases respectively (Yu et al., 2004).

Transcription at methylated sites can also be affected by methyl-CpG binding domain proteins (MBD) that are recruited and act as adaptor proteins between methylated DNA and chromatin modifying enzymes (Hendrich and Tweedie, 2003). The role of chromatin remodelling and histone modification as mechanisms involved in tumour and cancer development is only just emerging. Chromatin consists of approximately 2m of DNA coiled around a series of nucleosomes and is parcelled into the nucleus. Surprisingly, in this form chromatin retains a large degree of functionality (Richmond, 2006). The nucleosome consists of histones H2A, H2B, H3 and H4 as hetero-dimers that form a core unit. Histone tails carry a positive charge and are capable of interacting with the poly-anionic back bone of DNA contributing to nucleosome stability (Yap and Zhou, 2006). The regulation of chromatin structure and transcription is often influenced by post-translational modification of histone tail residues, affecting the accessibility of transcription factors to DNA. This includes methylation, acetylation, phosphorylation and ADP-ribosylation (Vitolo et al., 2000).

A specific group of transcription factors are involved in chromatin structure remodelling allowing other transcription factors access to DNA regions (Thomas and Chiang, 2006). Alterations in chromatin structure can also occur through ATP-dependent nucleosome remodelling (Johnson et al., 2005). Histone acetyltansferases (HATs), histone methyltransferases (HMTs) and histone deacetylases (HDACs) are all involved in a proposed aberrant histone modification signature for cancer (Fraga and Esteller, 2005). A large study by Ozdag et al. (2006) illustrated that six different cancer types, including adult GBMIVs, had a unique pattern of histone modifier gene expression compared to that of the corresponding normal tissue or other cancers.

The extent of non-coding RNA (ncRNA) involvement in epigenetics is largely unknown. Small regulatory ncRNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs) that are approximately 22 nucleotides in length and suppress translation in two ways. miRNAs undergo non-perfect pairing with target mRNA preventing translation and siRNAs pair perfectly with target mRNA causing degradation by the RNA-induced silencing complex. siRNAs are also involved in the silencing of chromatin and chromosome dynamics (Mattick and Makunin, 2006).

Recent discoveries suggest that the role of ncRNAs in disease is broader than initially understood, involving the control of gene expression through chromatin architecture and transcription (Mattick and Makunin, 2006). miRNA expression profiles have been used to classify human cancers (Lu et al., 2005) and disrupted miRNA target genes have been characterised in pituitary adenomas (Bottoni et al., 2007). Furthermore, miRNAs have been found to be disrupted in adult GBMIV through microarray analysis (Ciafre et al., 2005). In the primary GBMIV investigated, nine miRNAs were significantly up-regulated and four were significantly down-regulated. When the tumours were clustered according to the expression profile similarity of the miRNAs, a clear distinction could be seen between normal controls and GBMIV samples, suggesting a role for miRNAs in GBMIV development (Ciafre et al., 2005).

Control of transcription, translation and protein activation

The regulation of gene expression controls the amount of functional gene product available at a given time and when synthesis occurs. This process is modulated at all levels from transcription of mRNA to post-translational modification of proteins. This is essential, increasing the versatility and adaptability of cells, allowing specific proteins to be expressed in response to environmental change. Furthermore, gene regulation allows a multicellular organism to exist, utilising different transcriptomes each governed by different controls to influence cellular differentiation and morphogenesis.

The regulation of transcription controls when and how much RNA is synthesised. Specific mechanisms including DNA methylation, chromatin remodelling, histone modification and non-coding RNAs and their role in cancer have previously been discussed. Briefly, gene transcription by RNA polymerase is regulated by at least five

factors and mechanisms including, specificity factors that alter the specificity of RNA polymerase for a given promoter, repressors that bind to non-coding sequences on the DNA strand impeding the progression of RNA polymerase, general transcription factors that position RNA polymerase at the start of a protein-coding sequence and then release the polymerase to initiate transcription, activators that enhance the interaction between RNA polymerase and a particular promoter, facilitating gene expression and enhancers that are DNA sites bound to by activators that alter DNA structure, in order to join a specific promoter and initiation complex (Quivy et al., 2004; Wilusz and Wilusz, 2004; Valencia-Sanchez et al., 2006). The regulation of transcription involves several interactions between many transcription factors that allows for complex responses to multiple conditions in the environment. This permits spatial and temporal differences in gene expression.

Once DNA is transcribed additional mechanisms known as capping, splicing and the addition of a poly(A) tail control the amount of protein translated from the mRNA. Capping adds a 7-methylguanosine (m^7G) group to the 5' end, which protects the mRNA from 5' nuclease degradation. The cap also aids ribosomal binding. Splicing removes introns from the mRNA to ensure that only coding regions are translated into proteins and the addition of poly(A) tails to the 3' end increases the half life of mRNA (Mata et al., 2005; Wek and Cavener, 2007).

Posttranslational modification is the chemical modification of a protein after translation. This modification of amino acids include attaching the protein to other biochemical functional groups such as acetate, phosphate, various lipids and carbohydrates, changing the chemical nature of an amino acid or by making structural changes. Many alterations govern protein degradation influencing half life, enzyme affinity and efficacy and whether a protein is active or inactive (Mata et al., 2005; Wek and Cavener, 2007).

Disruption at any stage of transcription or translation impacts cellular pathways and is involved in tumourigenesis. Understanding the complex control mechanisms governing these processes will identify those pathways disrupted in specific tumours including astrocytoma, providing targets for new therapies (Lo et al., 2005).

Genetics and chromosomal alterations in paediatric astrocytoma

Histopathologically, paediatric astrocytoma are similar to adults. However, studies have shown that their biological behaviour is different to that of their adult counterparts. This suggests that these tumours have a different molecular pathogenesis. Studies investigating chromosomal aberrations in paediatric astrocytoma have identified alterations that also occur in adult astrocytoma and further changes that are common to paediatric astrocytoma alone. Furthermore, some paediatric high-grade astrocytoma show a normal karyotype, a characteristic uncommon in adult astrocytoma (Bigner et al., 1997; Warr et al., 2001; Rickert et al., 2001).

Cytogenetic analysis of paediatric PAI has shown that the majority of these tumours (>70%) have a normal karyotype (Bhattacharjee et al., 1997). Numerical and structural abnormalities of chromosomes 5, 6, 7, 8 and 9 have been reported, although definitive, non-random aberrations have not yet been identified (White et al., 1995; Zattara-Cannoni et al., 1998; Shlomit et al., 2000; Sanoudou et al., 2000b; Rickert and Paulus, 2004). Loss of the locus on chromosome 17p has been reported in some paediatric PAI studies but not consistently (von Deimling et al., 1993; Phelan et al., 1995; Willert et al., 1995). Whole chromosome alterations have been observed in paediatric PAI arising in patients above 10 years of age. The most common alterations were gain of chromosomes 5 and 7, present in 13% and 17% of cases, respectively. Whole chromosome alterations of chromosomes 4, 6, 9, 10, 11, 12 and 20 were present in 3% of cases and chromosome 15 in 6% of cases (Jones et al., 2006). Single nucleotide polymorphic (SNP) allelic arrays have also been used to identify regions of allelic imbalance in low-grade paediatric gliomas including 6 PAI. However, no detectable LOH was found at any of the 11562 SNP loci investigated in the PAI (Wong et al., 2006).

A study by Addo-Yobo et al. (2006) identified over-expression of *ErbB3* and *Sox10* in paediatric PAI. The ErbB family of proteins are thought to be involved in the growth and development of the normal nervous system. This study suggests that the up-regulation of *ErbB3* via *Sox10* in paediatric PAI, drives cell growth and proliferation, as previously seen in embryonic astrocytes (Britsch et al., 2001). PAI sensitivity to the tyrosine kinase inhibitor gefitinib is thought to occur via ErbB3.

The risk of developing PAI is increased in neurofibromatosis type 1 (NF1) patients. These patients have a mutation and functional loss of the *NF1* gene. Some sporadic paediatric PAI show LOH on chromosome 17q in the region of the *NF1* gene (Willert et al., 1995). Consequently, loss of *NF1* was expected to be a common event in these tumours, however this is yet to be established (Platten et al., 1996; Wimmer et al., 2002). At this time it is unknown why the loss of *NF1* increases the risk of PAI in NF1 patients.

In paediatric DAII, AAIII and GBMIV of patients below 15 years of age there is a correlation between karyotype complexity and tumour grade. DAII often have a normal karyotype or show simple chromosome changes compared to high-grade astrocytoma that show multiple complex chromosome aberrations frequently displaying hyperdiploid or polypoid karyotypes (Agamanolis and Malone, 1995; Bigner et al., 1997; Bhattacharjee et al., 1997; Roberts et al., 2001). Studies investigating diffuse astrocytoma in children show inconsistent findings. Specific studies describe chromosome alterations that are common in adult astrocytoma including -17p, +7, -10 and -22 (Schrock et al., 1996; Bhattacharjee et al., 1997). Other studies describe different alterations to those seen in adults (Neumann et al., 1993; Agamanolis and Malone, 1995; Cheng et al., 1999).

Chromosomal regions that have shown copy number aberrations in malignant astrocytoma from patients below 25 years of age include -16p, -17p, -19p, -22, +2q, +12q, +13, +4q, +5q and +8q. Seven regions of high copy number amplification were observed in the same study at 8q21-22 (three cases), 7q22-23 (two cases) and 1p21-22, 2q22, 12q13-pter, 12q15-21, and 13q11-14 (one case each) (Warr et al., 2001). Rickert et al. (2001) also identified gain of 1q, 3q and 16p and loss of 8q and 17p in paediatric GBMIV. Furthermore, in paediatric AAIII different aberrations have been reported involving gain of 5q and loss of 6q, 9q, 12q and 22q (Rickert et al., 2001). Moreover, LOH on 22q was found to occur at the same frequency in paediatric astrocytomas as seen in adults (Nakamura et al., 2007). Interestingly, the same study found a higher frequency of *TP53* mutations and LOH on chromosomes 19q and 22q in tumours from children above 6 years of age at diagnosis, compared with those from younger children (Nakamura et al., 2007).

Amplification of *EGFR* is not commonly found in paediatric astrocytoma as seen in adults (Kraus et al., 2002; Khatua et al., 2003; Pollack et al., 2006). However, protein over-expression has been reported in 80% of paediatric astrocytoma (Bredel et al., 1999). Moreover, a recent study investigating a small number of high-grade paediatric astrocytoma identified up-regulation of *EGFR* expression and other genes linked to angiogenesis (Khatua et al., 2003).

TP53 mutations have been found in paediatric astrocytoma at varying levels and at a much lower frequency than in adult astrocytoma (Sure et al., 1997; Cheng et al., 1999; Hayes et al., 1999; Pollack et al., 2002). However, in a study by Pollack et al. (2002) *TP53* mutations were found in paediatric GBMIV at the same rate as previously found in adults. The same study also correlated TP53 protein over-expression with increasing paediatric astrocytoma grade. Furthermore, high-grade astrocytomas in children above 3 years of age showed a *TP53* mutation rate comparable to malignant astrocytoma from young adults (Pollack et al., 2001). This positive correlation between increasing *TP53* mutation rate and paediatric astrocytoma patient age has also been identified by Nakamura et al. (2007) High-grade astrocytoma from children above 6 years of age showed an increase in *TP53* mutation frequency compared to those below 6 years of age. Although no amplification of the *MDM2* gene has been reported, over-expression has been found in 67% of paediatric GBMIV (Sung et al., 2000).

In paediatric GBMIV, homologous deletion of the *CDKN2A* locus at 9p21 was found in 10% of tumours and mutations of this gene were also found in 8% and 13% of AAIII and GBMIV respectively (Sure et al., 1997; Raffel et al., 1999; Newcomb et al., 2000) Furthermore, loss of *CDKN2A* expression was found in 60% of paediatric GBMIV (Sure et al., 1997). It has been reported that disruption of the TP53 pathway through one or more of these mechanisms occurs in 95% of paediatric GBMIV, a figure similar to that of adult astrocytoma. However, the Rb pathway is only disrupted in 25% of paediatric GBMIV, a much lower frequency than that seen in adult GBMIV (Sung et al., 2000). Mutation of the *PTEN* gene has been linked to poor prognosis in paediatric astrocytoma, but this has been reported in only a few cases (Raffel et al., 1999; Kraus et al., 2002; Pollack et al., 2006).

Stem cells and brain tumours

Tumours do not consist of a single cell type, but are composed of a variety of cells with different phenotypic and genotypic characteristics (Nakano and Kornblum, 2006). Traditional thinking suggests that many cells within a tumour are capable of giving rise to another tumour cell (Reya et al., 2001). In contrast, the cancer stem cell hypothesis suggests that tumours arise from a minority cell type with self renewing properties, which are capable of giving rise to other more differentiated cell types. This hierarchy of cell origin has been well documented in leukaemia (Bonnet and Dick, 1997). From this hypothesis it was proposed that cancer stem cells originate from normal adult or tissue specific stem cells that have acquired mutations. Although this has not been proven, there is some evidence to suggest this in leukaemia. The implications of these suggestions are that target therapies would need to be aimed at cancer stem cells and not cellular derivatives to have any chance of success (Nakano and Kornblum, 2006).

Several studies have investigated the cancer stem cell hypothesis in adult GBMIV. A sub-population of cells have been characterised with increased tumorigenic potential and expression of *CD133*, a marker of neural precursor cells (Singh et al., 2004). The $CD133^+$ population of cells showed consistent stem cell characteristics including expression of stem cell markers *CD133*, *Sox2*, *Musashi* and *Nestin*, multi-lineage differentiation, with expression of *GFAP* and *S100β* (astrocytes), *Map-2* and *TUJ1* (neurons) or *O4* and *GalC* (oligodendrocytes) and neurosphere formation. The cells were also found to be highly tumorigenic in immunocompromised mouse brains compared to $CD133^-$ cells (Bao et al., 2006).

Furthermore, the $CD133^+$ adult GBMIV cells were found to be highly resistant to ionizing radiation compared to the $CD133^-$ GBMIV cells. This was attributed to the efficiency of DNA repair mechanism induction in the $CD133^+$ cells. Moreover, after radiation, the $CD133^+$ fraction of the tumour was increased. Ionising radiation has been the primary treatment for GBMIV for the last 40 years, but has become a temporary solution, reducing tumour size rather than a cure. These results provide an explanation for GBMIV response to ionising radiation and suggests that future therapies may need to target the $CD133^+$ tumour cell population (Bao et al., 2006).

A second study by Piccirillo et al. (2006) identified bone morphogenetic proteins (BMPs), specifically BMP4, as factors that can reduce the stem-like, tumour initiating $CD133^+$ tumour cell population. BMPs play a role in promoting the differentiation of adult stems to astrocytes (Panchision and McKay, 2002). $CD133^+$ adult GBMIV cells expressing BMP4 showed reduced proliferation in response to mitogens, increasing the number of cells in the G0/G1 phase of the cell cycle and simultaneously decreasing the number of cells in the S phase. In addition, morphological changes were seen in the cells including increased *GFAP* expression, decreased *MAP5* and *βIII-tubulin* expression (neuronal markers) and decreased *GalC* expression. Overall, BMP4 expression was shown to promote differentiation in adult GBMIV $CD133^+$ cells, reducing the pool of tumour initiating cells (Piccirillo et al., 2006). These results also imply that GBMIV cell populations may retain stem cell hierarchy and the ability to respond to the normal signals that induce them. This also fuels interest in developing therapies that promote tumour cell differentiation (Dirks, 2006).

Expression microarray studies investigating astrocytoma

Several microarray studies have been carried out on astrocytoma (Huang et al., 2000; Rickman et al., 2001; Fuller et al., 2002; Kim et al., 2002b; Nutt et al., 2003; Shai et al., 2003; van den et al., 2003; Godard et al., 2003; Mischel et al., 2003; Rorive et al., 2006). The studies are difficult to compare for several reasons. Initially, various array platforms have been used to generate gene expression profiles, consequently different probes and numbers are used to detect the same gene. Secondly, data analysis programmes for each platform are varied, as are the statistical tests and bioinformatic approaches. The studies use different types and numbers of astrocytoma, as well as control tissues or samples. Overall, more studies are needed and standards need to be introduced to allow comparisons between different data sets and results.

The studies involving adult astrocytoma have revealed that tumour tissue expression profiles significantly differ from that of normal tissue. Sub-groups of astrocytoma grades have also been recognised and distinct genetic profiles may be used to aid diagnosis and predict tumour behaviour. Eventually, this could lead to new tailored treatments determined by astrocytoma sub-type and expression profile.

Results collected from these studies correlated with previous findings in adult astrocytoma. A study by Mischel et al. (2003) highlighted increased VGF and/or receptor expression in high-grade tumours as expected. Furthermore, larger studies have highlighted a number of novel genes aberrantly expressed in astrocytoma. A study of adult DAII highlighted novel differentially expressed genes including *AAD14, SPARC, LRP, PTN, hBAP* and *IFI9-2* that were up-regulated, whilst *CLK, TDGF1, BIN1, GAB1, TYRO3, LDH-A, adducing 3, GUK1, CDC10,* and *KRT8* were down-regulated. Furthermore, *TIMP3, MYC, EGFR, DR-nm23, nm23H4* and *GDNPF* were only expressed in the tumour samples compared to controls (Huang et al., 2000). Quantitative real-time polymerase chain reaction (Q-PCR) was used in the majority of cases to validate aberrant expression results found in the microarray studies.

Only three microarray studies have investigated paediatric astrocytoma, with two focusing on PAI (Khatua et al., 2003; Wong et al., 2005; Sharma et al., 2007). The study by Wong et al. (2005) analysed 21 paediatric PAI using the Affymetrix GeneChip® U133A array. A total of 428 genes showed a 3-fold and significant change in expression from the normal; 192 up-regulated and 236 down-regulated. Of the 428 genes that showed differential expression, 96 were sub-grouped according to biological processes. A total of 23 genes were involved in neurogenesis; 20 genes in cell adhesion; 14 genes in synaptic transmission; 11 genes CNS development; 10 genes in potassium ion transport; 10 genes in protein amino acid dephosphorylation; and 8 genes in cell differentiation. This study also identified two sub-groups of paediatric PAI within the 21 samples. A group of 89 genes were differentially expressed between the two sub-groups. The sub-groups were further distinguished by immunohistochemical staining of myelin basic protein (MBP). Paraffin sections of 8 of 10 PAI in one sub-group revealed fewer positively stained cells which correlated with increased disease progression in these PAI. The study suggests that *MBP* may be used as a marker to distinguish PAI with increased risk of disease progression.

A second study focused on 41 PAI ranging in age between 1 and 53 years, which were a mixture of sporadic PAI and those arising in patients with NF1. In this investigation, the Affymetrix GeneChip® U133 Plus 2 array was used to investigate differential gene expression. No gene expression signature was identified which could discriminate those

PAI that were clinically aggressive, recurred, occurred sporadically or were from patients with NF1. Whole-genome expression analysis could not segregate the tumours based on tumour or patient parameters. However, a group of 36 genes were shown to be differentially expressed between supratentorial tumours and those located in the posterior fossa (Sharma et al., 2007).

Khatua et al. (2003) distinguished low and high-grade paediatric astrocytoma using genes involved in angiogenesis. The *EGFR/FKBP12/HIF-2α* pathway was found to be over-expressed in high-grade tumours. This study used the Affymetrix GeneChip® U95Av2 array containing 12000 genes and selected 133 genes involved in angiogenesis for detailed analysis. In total, 44 genes were differentially expressed between low and high-grade astrocytoma. Hierarchical clustering using these genes, showed that the tumours clustered according to grade. This study showed that the over-expression of specific *EGFR/FKBP12/HIF-2α* pathway genes in high-grade paediatric astrocytoma promotes angiogenesis and could be used as target genes for new therapies.

aCGH studies investigating astrocytoma

Analogue CGH (comparative genomic hybridisation) has previously been used to identify common chromosome CNAs in paediatric astrocytoma. However, this technique is less sensitive than the 1MB resolution of aCGH (array comparative genomic hybridisation). Several aCGH studies have recently defined genome wide aberrations at a resolution of 1Mb in adult malignant astrocytoma (Cai et al., 2002; Nigro et al., 2005; Rossi et al., 2005; Misra et al., 2005; Korshunov et al., 2006; Ruano et al., 2006; Vranova et al., 2007; Wrensch et al., 2007). Only one study investigated paediatric astrocytoma, specifically PAI in children and young adults (Jones et al., 2006). An additional study used SNP allele arrays to investigate genome-wide allelic imbalances in low and high-grade paediatric astrocytoma (Wong et al., 2006).

Potential genetic sub-groups were identified in two studies investigating adult GBMIV (Misra et al., 2005; Korshunov et al., 2006). The sub-groups did vary, but both studies identified loss of chromosome 10 and gain of chromosome 7 as the most common chromosome aberrations in adult GBMIV. Other single clone alterations were identified by the studies, which mapped to regions containing putative oncogenes and tumour

suppressors. These clones were also used to define the genetic sub-groups, providing a more detailed and specific characterisation of adult GBMIV and the key regions and genes disrupted in this tumour. The study by Misra et al. (2005) included 50 primary GBMIV biopsy samples that clustered into 3 distinct groups according CNAs. Group A contained 23 GBMIVs with no consistent aberrations compared to groups B and C that consisted of 9 GBMIV with loss of chromosome 10 and 18 GBMIV with loss of 10 and gain of 7 respectively. The authors propose that the tumours of each group would show varying responses to different treatment regimes given the diverse CNAs identified in each cluster. No correlations were made between survival and specific CNAs or groups but only 3 patients involved in the study were considered to be long term survivors, which prevented this approach (Misra et al., 2005).

In comparison Korshunov et al. (2006) investigated 70 GBMIV and identify 46 CNAs that were associated with patient outcomes, 26 were associated with shortened survival and 20 correlated with good prognosis. Hierarchical cluster analysis distinguished two genetically distinct groups of GBMIV consisting of 56 (group 1) and 14 (group 2) tumours, respectively that showed a significant difference in survival. The 5-year survival rate was 0% for group 1 and 63% for group 2. The authors suggest that aCGH screening of GBMIV could provide clinically useful information and potentially improve the quality of treatment (Korshunov et al., 2006). Both of these studies consist of large sample numbers that have allowed hypotheses to be statistically proved. However both studies could benefit from addition sample numbers with broader tumour and patient parameters including varied survival rates.

Jones et al. (2006) investigated 32 PAI in patients below 15 years of age and 12 PAI in patients above 15 years of age. Overall, 64% of tumours had a normal karyotype, with 32% of tumours showing gain of whole chromosomes. Whole chromosome alterations were only observed in tumours arising in patients above 10 years of age, the most common of which were gain of chromosomes 5 and 7, occurring in 13% and 17% of cases respectively. Alterations of chromosome 15 was present in 6% of cases followed by chromosomes 4, 6, 9, 10, 11, 12 and 20 that were seen in 3% of cases. Loss or gain of single BAC clones are still to be confirmed in this study.

Short-term cell cultures and the study of astrocytoma

The use of short-term cell cultures and established cell lines in the study of cancer has been constantly criticised. Difficulties have been encountered when characterising cell lines due to the changes in cellular environment including the promotion of proliferation *in vitro* compared to *in vivo*, reduction in cell – cell and cell – matrix interactions due to purified culture fractions and loss of three-dimensional architecture and alterations in nutrient and hormonal availability. The culture environment promotes spreading, migration and proliferation and consequently these factors will induce changes in gene expression within *in vitro* cells compared to those *in vivo*.

A recent study by Camphausen et al. (2005) used microarray analysis to characterise two human glioma cell lines (U251 and U87) when grown *in vitro* and *in vivo* subcutaneously or as intracerebral xenografts. For each cell line, the gene expression profiles generated from each approach were significantly different suggesting that the *in vivo* growth environment modulates gene expression compared to orthotropic growth conditions, which induce different gene expression changes (Camphausen et al., 2005). These data also suggests that tumour cells respond to the environment in which they are grown consistently, inducing similar gene expression changes. Moreover, a study by Ross et al. (2000) characterised systematic variation in gene expression in 60 human cancer cell lines originating from 9 different types of solid tumour. Classification of the cell lines highlighted an association to the tumours of origin from which the cell lines were derived. Specific features of the gene expression profile also correlated with physiological properties of the cell lines including doubling time in culture, drug metabolism or the interferon response. Furthermore, a branch of the hierarchical clustering tree included all GBMIV, renal cell carcinoma and carcinoma derived cell lines. Those genes that specifically characterised this group of cultures included many involved in stromal cell functions. It was also noted that the GBMIV cell lines showed mesenchymal characteristics and may explain the ease of establishing these cell lines (Ross et al., 2000).

Both studies highlight that changes in gene expression do occur in tumour derived cell cultures but that the gene expression changes are different depending on the growth environment yet consistent. The clustering of cultures from the same tumour of origin confirms that the cultures do maintain characteristics of the tissue of origin. This also

suggests that providing that the culture environment is maintained, comparisons could be made between cultures derived from different grades of the same tumour, or specific tumour types in order to study specific tumour characteristics or specific grades.

AIMS

Little is known about the genetics of paediatric astrocytoma. The majority of these tumours have normal karyotypes and few common genomic alterations have been recognised. A number of studies have included the investigation of gene or protein expression but show conflicting results. Furthermore, the common genomic and expression alterations seen in adult astrocytoma are comparatively rare in paediatric astrocytoma.

The initial aim of this study was to identify differentially expressed genes in different grades of malignancy in paediatric astrocytoma biopsy samples. The samples for this study were frozen biopsy tissues collected from consenting patients at Great Ormond Street Hospital between 1980 and 2005. Unfortunately, it was not possible to obtain total RNA from paediatric DAII, AAIII and GBMIV tissues of sufficient quality for expression microarrays, due to tissue storage procedures in the 1980's and early 1990's. However, short-term cell culture samples were available from the same tumours and consequently this sample source was used as an alternative to investigate differentially expressed genes in paediatric astrocytoma.

As a result the aims of this study were to;

- Identify differentially expressed genes in paediatric astrocytoma using microarray technology and identify expression profiles unique to paediatric astrocytoma that may correlate with tumour or patient parameters.

- Correlate differentially expressed genes with cellular pathways that may be involved in tumour development.

- Identify copy number alterations in paediatric astrocytoma using aCGH and correlate these with differential gene expression as a mechanism of gene expression alteration.

- Investigate methylation as a mechanism causing differential gene expression in paediatric astrocytoma.

- ❖ Compare the use of biopsy and short-term cell cultures in the study of paediatric astrocytoma and the possible influence of short-term cell culture on gene expression.

- ❖ Investigate the use of control samples in microarray experiments and the study of paediatric astrocytoma with regards to biopsy and short-term cell culture tumour samples and microarray experimental approaches.

- ❖ Investigate the accuracy of single microarray chips for individual tumour sample study, by comparing chip replicates of a small number of samples.

CHAPTER 2

MATERIALS AND METHODS

The Local Research Ethics Committee at the Institute of Child Health and Great Ormond Street Hospital gave approval for this study (Study REC number 04/Q0508/98).

Additional work not directly required for the understanding and interpretation of the results can be found in the Appendix or on the Supplementary Data CD included at the back of this thesis.

MATERIALS

Information regarding reagent suppliers and buffer preparations are detailed in Appendix I. Plastics were all purchased from Scientific Laboratory Supplies or Triple Red and all chemicals were obtained from Sigma Aldrich unless otherwise stated.

Samples

Astrocytoma biopsy samples were collected from consenting patients at Great Ormond Street Hospital between 1980 and 2005. The samples were obtained from tissue removed during surgery, surplus to diagnosis. The surgeons carrying out this procedure were Mr W. Harkness, Professor R. Hayward and Mr D. Thompson. All tumour samples were directly adjacent to tumour tissue processed for routine histological evaluation and were first examined macroscopically to ensure that no frankly normal tissue was present. They were diagnosed according to the WHO classification and reviewed by two neuropathologists Dr B. Harding and Dr T. Jacques (Kleihues and Cavenee, 2000).

In 2007, the fourth edition of the WHO Classification of Central Nervous System Tumours was published. In this edition, the WHO grading scheme has been up-dated to include new entities and histological variants where appropriate, as well as tumour genetic profiles (Louis et al., 2007b). A new astrocytoma variant was included in the edition, termed pilomyxoid astrocytoma, as previously described in the Introduction. The new grading scheme for astrocytomas was described by Louis et al. This account

states that WHO defines diffusely infiltrating astrocytic tumours with cytological atypia alone as grade II, those showing anaplasia and mitotic activity as grade III and tumours additionally showing microvascular proliferation and/or necrosis as WHO grade IV. Furthermore, this account also states that the finding of a solitary mitosis in a sample specimen does not confer grade III behaviour and that separation of grade II and III tumours may be more reliably achieved by determination of MIB-1 labelling indices. For grade IV, some authors accept only the criterion of endothelial proliferation, i.e. an apparent multi-layering of endothelium (Louis et al., 2007b).

The initial diagnoses of tumours included in this study, have been reviewed in light of the 2007 WHO classification grading scheme. It was not possible to review all cases and some cases could only be reviewed from case notes and pathology reports. Consequently, caution should be taken when acknowledging the new diagnoses in these cases. Interestingly, the majority of initial diagnoses that were PAI and GBMIV remained the same according to the 2007 WHO grading scheme. However, all initial diagnoses of DAII were altered under the new scheme, the majority of which were deemed PAI. As this review was completed at the end of the study, initial diagnoses and those made in 2007 are referred throughout the report. If two conflicting diagnoses have been reported for a tumour, the diagnosis shown in bold corresponds to the WHO grading scheme of 2007.

Short-term cell culture preparations were set up in the laboratory from biopsy tissue immediately after receipt. At the time of this study, the majority of tumour biopsy and short-term cell culture samples had already been collected and stored in liquid nitrogen. Details of all samples and patients used in this study are given in Tables 2.1, 2.2 and 2.3.

Normal human adult and foetal brain total RNA from whole brain and specific brain regions were obtained as pooled samples where possible from several commercial companies including AMS Biotechnology, United States Biologicals and BD Biosciences. Foetal astrocytes were obtained from Cambrex Bio Sciences and Professor G. Pilkington, at Portsmouth University (Table 2.1).

Table 2.1 Data of the normal control samples in this study.

Ref[1]	Origin[2]	Location[3]	Number of pooled donors [4]	Sex[5]	Age range[6]
A TB	Young Adult	Total Brain	5	M	24-30 yrs
A TB 34	Young Adult	Total Brain	3	M	21-27 yrs
A TB 35	Young Adult	Total Brain	3	M	23-29 yrs
A TB 36	Young Adult	Total Brain	3	M	22-28 yrs
A Cere	Adult	Cerebellum	24	M & F	16-70 yrs
A CorC	Adult	Corpus Callosum	24	M & F	24-68 yrs
F TB	Foetus	Total Brain	21	M & F	26-40 wks
F Cere L	Foetus	Cerebellum (Left)	1	F	37 wks
F Cere R	Foetus	Cerebellum (Right)	1	F	33 wks
F CorC	Foetus	Corpus Callosum	3	M & F	22-36 wks
GP-9	Foetal Astrocytes	-	-	-	-
NHA	Foetal Astrocytes	-	-	-	-

Ref[1]: Reference name and number; Origin[2]: Origin of RNA; Location[3]: Region of brain tissue used for RNA isolation; Number of Pooled Donors[4]: Number of donors pooled in each sample; Sex [5]: M = male F = female; Age Range[6]: Age range of patients pooled in each sample.

Characterisation of short-term cell cultures

Short-term cell cultures were derived from tumour tissue directly adjacent to that processed for routine histological evaluation and examined macroscopically to ensure that no frankly normal tissue was present. Cell morphology was monitored throughout cell culture to ensure cells maintained an astrocytic phenotype. This included small rounded cells and spindle dendritic-like cells as characterised by Wang et al. (2007). During early passage, cells of some of cultures showed cytoplasmic processes that contacted each other as seen by Ishiwata et al. (2004).

CNAs were also investigated in each culture, as discussed in Chapter 5, as genetic instability and abnormal karyotypes are common in tumorigenic cells. However, not all paediatric astrocytoma exhibit CNAs as previously discussed in the Introduction. The gene expression profile of each short-term culture was also investigated and deemed to be significantly different from the normal as discussed in Chapter 5. Doubling times and *TP53* and *P73* mutations have previously been investigated in these short-term cultures although the data is not discussed in this study. Cell surface markers were not investigated during or prior to this study. All short-term cultures investigated in the study were considered to be tumorigenic.

Table 2.2 Clinical and pathological data of the astrocytoma in this study.

IN[1]	Age[2]	Sex[3]	Grade at diagnosis[4]	Grade as of 2007[5]	Location[6]	Survival[7]
1350	4.3	M	PAI	-	Posterior Fossa	Unknown
1520	7	M	PAI	PAI	L. Temporal Parietal	DIS 112 (A)
1524	13	M	PAI	PAI	L.Cerebellum	13 (A)
1591	2	F	PAI	**PAI**	Frontoparietal	60 (D)
1740	8	M	PAI	**PAI**	Posterior Fossa	160 (A)
2017	7	M	PAI	**PAI**	Posterior Fossa	124 (A)
2110	2.7	M	PAI	PAI	Posterior Fossa	DIS 125 (A)
2122	10	F	PAI	PAI	Supraseller Optic Chiasm	DIS 78 (A)
2356	4	M	PAI	PAI	Cerebellum	142 (A)
2368	8	F	PAI	PAI	Posterior Fossa	DIS 87 (A)
2524	4	F	PAI	PAI	Midline-Posterior Fossa	DIS 39 (A)
2604	6	F	PAI	-	Optic Chiasm	Unknown
2631	4	F	PAI	PAI	Cerebellum	21 (A)
2674	5	M	PAI	PAI	L.Cerebellum	112 (A)
2688	5	M	Low	PAI	Cerebellum	55 (A)
2788	9	M	PAI	PAI	Posterior Fossa	45 (A)
2825	2.75	F	PAI	PAI	Posterior Fossa	104 (A)
2921	3	M	PAI	PAI	Posterior Fossa	48 (A)
2940	1.75	M	PAI	PAI	Posterior Fossa	74 (A)
2969	3.5	F	PAI	PAI	Posterior Fossa	57 (A)
3013	11.5	M	PAI	**PAI**	Posterior Fossa	58 (A)
3017	8.25	F	PAI	PAI	Posterior Fossa	59(A)
3115	3.5	F	PAI	PAI	Optic Pathway	33 (A)
3126	9	M	PAI	PAI	Posterior Fossa	31 (A)
3156	1.9	F	PAI	PAI	Posterior Fossa	1 (A)
380	1.8	F	DAII	-	Posterior Fossa	92 (D)
1145	11	M	DAII	-	Midline Cerebellar	60 (A)
1751	6	F	DAII	PAI	R. Temporal	63 (A)
1869	15	F	DAII	**PAI**	Posterior Fossa	139 (A)
1930	13	F	DAII	AAIII	Temporal	53 (A)
2012	7	F	DAII	**PAI**	L. Temporal	145 (D)
2044	9	M	DAII	PAI	Posterior Fossa	DIS 18 (A)
2102	8	M	DAII	PAI	Brainstem	67 (A)
3032	9.5	F	GA	AAIII	L.Thalamic	12 (D)
2003*	9	F	DAII	PAI	R. Temporal	117 (A)
2591*	12	F	DAII	PAI	Supratentorial	117 (A)
178"	13	M	GBMIV	-	L.Temporal	3 (D)
179"	13	M	GBMIV	-	Base of Skull	3 (D)
699	15	M	GBMIV	-	R.Thalamus	Unknown
1163	0.2	M	GBMIV	-	R.Parietal	DIS 153 (A)
1262	14	M	GBMIV	-	L.Parietal	12 (A)
1419**	8.5	F	GBMIV	GBMIV	Supratentorial	12 (D)
1495	6	F	GBMIV	GBMIV	Supratentorial	80 (A)
1523	10	F	GBMIV	**GBMIV**	L.Parietal	33 (D)
1566	6.4	F	GBMIV	**GBMIV**	Posterior Fossa	7 (A)
2087	3	M	GBMIV	**GBMIV**	Brainstem	(D)
2675	14	M	GBMIV	GBMIV	Brainstem	16 (D)
3046	15.9	M	GBMIV	AAIII	L.Frontal	14 (D)

IN[1]: Institute of Neurology assigned number; Age[2]: Age in years at diagnosis; Sex[3]: M = Male; F = Female; Grade[4/5] PAI = pilocytic astrocytoma; DAII = diffuse astrocytoma; GA = gemistocytic astrocytoma; AAIII = anaplastic astrocytoma; GBMIV = glioblastoma multiforme; Low = low grade tumour. It was not possible to review all cases in this study. Furthermore, it was only possible to review the case notes and pathology report for specific cases and not slides (these tumours are shown in bold). Location[6]: L = left; R = right; Survival[7]: survival in months from the date of diagnosis, A = alive; D = deceased. DIS = Patient has been discharge; * and " = tumours from the same patient; ** = patient diagnosed with Turners Syndrome (partial or complete loss of chromosome X). Biopsy tissue was available from tumour samples highlighted blue. Short-term cell culture preparations were available from tumour samples highlighted green. Both biopsy and short-term cell culture preparations were available for samples highlighted red.

Table 2.3 Clinical and pathological data of the adult astrocytoma in this study.

IN[1]	Age[2]	Sex[3]	Grade[4]	Location[5]	Survival[6]
2435	57	F	GBMIV	R. Parietal Lobe	Unknown
2731	51	M	GBMIV	R. Occipital Parietal Mass	Unknown
2810	66	M	GBMIV	L. Frontal Intrinsic Lesion	Unknown
1760	57	F	GBMIV	R. Parietal Lobe	12 (D)
1265	70	F	GBMIV	R. Parietal Lobe	15 (D)
1528	61	M	GBMIV	R. Occipital	7 (D)
2234	61	F	GBMIV	Temporal Lobe	Unknown

IN[1]: Institute of Neurology assigned number; Age[2]: Age in years at diagnosis; Sex[3]: M = Male; F = Female; Grade[4]: GBMIV = glioblastoma multiforme; Location[5]: L = left; R = right; Survival[6]: survival in months from the date of diagnosis, A = alive; D = deceased. Short-term cell culture preparations were available from tumour samples highlighted green. Both biopsy and short-term cell culture preparations were available for samples highlighted red. All tumours are adult primary GBMIV.

METHODS

Biopsy tissue preparation

Excess tumour biopsy tissue for research purposes was immediately transferred into Hams F10 nutrient mix (Invitrogen Ltd) containing additional antibiotics, penicillin/streptomycin (100IC/ml / 100µg/ml), kanamycin (50µg/ml) and amphotericin (2.5µg/ml). The biopsy tissue was stored at 4°C for immediate collection from the operating theatre. Rapid collection and processing was essential to obtain suitable tissue for short-term cell culture preparations and DNA and RNA extractions.

A sterile class II laminar flow cabinet (ICN Pharmaceuticals) was used for biopsy tissue preparation. Initially, the tissue was removed from the media and placed in a sterile petri dish. Where possible, 100mg of tissue was prepared in RNAlater™ (Qiagen) and frozen in liquid nitrogen for RNA extraction. Approximately 10mg of tissue was used for short-term cell culture preparations and the remaining tissue was frozen in liquid nitrogen for DNA extraction.

Tissue for short-term cell culture preparations was diced using crossed sterile scalpels and transferred to a sterile 30ml sterile universal tube containing 2ml media (Hepes buffered Hams F10 nutrient mix and 10% foetal calf serum (FCS) (Invitrogen Ltd)) and 1ml of collagenase solution (2000U/ml). The tissue was incubated at 37°C for a minimum of 1 hour to allow collagenase breakdown of the tissue structure. Next, 7ml of media was added and the preparation and centrifuged (Wifug 500E, DJB Labcare) at 1,000rpm for 5 minutes. The supernatant was removed, the remaining pellet re-

suspended in 10ml of media and placed in a 25cm^2 cell culture flask. The sample was incubated in a non-CO_2 incubator at 37°C and checked regularly for growth.

Cell culture

All cell culture procedures took place in a sterile class II laminar flow cabinet. During cell culture procedures, 70% methanol was used to clean the cabinet and wipe any objects entering the cabinet. Details of media, serum, antibiotics and trypsin can be found in Appendix I.

Maintaining cells in culture

Cells in culture were fed once a week or when the media changed from pink to yellow. This change indicates a reduction in nutrient content, an increase in cell waste products due to cell growth and a drop in pH. Media was aspirated from the culture flask and replaced; 25cm^2 flasks with 10ml of media, 75cm^2 flasks with 13ml of media and 150cm^2 flasks with 35ml of media.

Passaging cells

Once cells were confluent they were passaged to a larger flask or flasks according to the subcultivation guidelines below. This maintained sufficient tumour cell density allowing population expansion. Upon each passage using trypsin (Invitrogen Ltd), the cell passage number was increased by one. Initially, the media was aspirated and the cells washed at least once with hanks buffered saline solution (HBSS) (Invitrogen Ltd). To detach the cells from the flask, 3ml of trypsin was added and the flask incubated at 37°C for approximately 10 minutes. After incubation, 7ml of media was added to inactivate the trypsin and the flask contents transferred to a sterile universal. The universal was centrifuged at 1,000rpm for 5 minutes in order to pellet the cells. Following removal of the supernatant, the cell pellet was re-suspended in 10ml of fresh media and transferred to the necessary flask or flasks.

- 1 x 25cm^2 flask to 1 x 75cm^2 flasks
- 1 x 75cm^2 flask to 3 x 75cm^2 flasks or 1 x 150cm^2 flask
- 1 x 150cm^2 flask to 3 x 150cm^2 flasks

Freezing cells in liquid nitrogen

Freezing of cultured cells in liquid nitrogen creates a stock of each tumour sample. Once the cells were trysinised and re-suspended in 10ml of fresh media, as previously described, 0.4ml of the suspension was added to 19.6ml of Isoton II (Beckman Coulter UK Ltd) and counted using a Coulter Counter (Beckman Coulter). The remaining suspension was centrifuged at 1,000rpm for 5 minutes to pellet the cells. The supernatant was removed and the cells re-suspended in a volume of FCS containing 10% dimethylsulphoxide (DMSO) to reach a final concentration of 1 million cells per 1ml. Finally, 1ml of the suspension was transferred to a cryovial and stored at -70°C for 24 hours. The vials were then placed in liquid nitrogen storage.

Thawing frozen cell stocks

Initially, a vial of cells was taken from liquid nitrogen, placed in warm water at approximately 37°C and allowed to thaw. The cell suspension was removed from the vial and pipetted very slowly into a universal containing 10ml of media. The universal was then centrifuged at 1,000rpm for 5 minutes and the supernatant removed leaving a cell pellet. This was re-suspended in 10ml of fresh media and transferred to a $25cm^2$ flask. The flask was placed in an incubator at 37°C to allow the cells to attach to the flask surface. The media was then changed after 24 hours.

Mycoplasma testing and treatment

The MycoAlert™ mycoplasma detection assay (Cambrex Bio Science) was used to test all short-term cell cultures for the presence of the prokaryote mycoplasma. If a short-term cell culture was found to be contaminated, a treatment regime was completed prior to any further work, to remove the prokaryote. Subsequent cultures were kept separate from initial cultures until the contamination had been removed.

To complete the MycoAlert™ test, 5ml of media was removed from cultures that were 80% confluent. This was centrifuged at 1,000rpm for 5 minutes to remove any cell debris. From this media, 0.1ml was pipetted into a 96 well plate. The addition of the test reagent initiated mycoplasma lysis, releasing specific enzymes which reacted with the test substrate, converting ADP to ATP. Luminometry based detection of this ADP-ATP conversion, using the Mediators PhL plate luminometer (Auren Biosystems GmbH),

indicated mycoplasma infection. Mycoplasma contaminated cultures were treated with Plasmocin™ (Autogen Bioclear UK Ltd) at 25μg/ml for 2 weeks.

Mycoplasma contaminated cell cultures show altered gene expression profiles, compared to uncontaminated cultures of the same cells. This is temporary and once the contamination is removed gene expression returns to the original level (Miller et al., 2003). As this study involved the investigation of paediatric astrocytoma gene expression profiles it was essential that any cultures used were free of mycoplasma.

RNA

All equipment and surfaces were cleaned with RNase *AWAY* (Molecular BioProducts Inc.) prior to any RNA work. All tubes and pipette tips were RNase/DNase free and any water used was either RNase/DNase free (supplied with most Qiagen Kits) or DEPC-treated.

RNA extraction from biopsy tissue

All RNA extractions were competed in a sterile Astecair 5000E hood (Bioquell UK Ltd). Between 50-100mg of tissue was used for each extraction depending on the initial tissue size. The biopsy samples were taken from liquid nitrogen storage and allowed to thaw on ice. Once thawed the tissue was weighed, cut and transferred to a sterile glass pestle and mortar containing 1ml of TRIzol® Reagent (Life Technologies). The tissue was homogenised and incubated to ensure all nucleases had been denatured. Phase separation was used to isolate the RNA using chloroform according to the manufacturer's instructions. The resulting RNA pellet was re-suspended in 0.1ml of RNase DNase-free water.

The RNeasy® Mini Column Kit (Qiagen) was used to clean the extracted RNA following the manufacturer's instructions. Briefly, 0.35ml of RLT buffer (containing β-mercaptoethanol at 0.01ml per 1ml of RLT) was added to the re-suspended RNA and mixed by gentle vortexing. A further 0.25ml of absolute ethanol was added and mixed via pipetting. The total mixture was transferred to an RNeasy® mini column and centrifuged for 15 seconds at 10,000rpm. Two wash steps were completed, the column transferred to a new collection tube and 30μl of RNase/DNase-free water added to the

membrane. The column was then incubated at room temperature for 1 minute prior to centrifugation at 10,000rpm for 1 minute. The quantity and quality of the eluted RNA was assessed before storage at -80°C.

RNA extraction from cell cultures

Initially, cells that were 70% confluent in three $150cm^3$ flasks were detached using trypsin and counted using the Coulter Counter as previously described. Qiagen RNeasy® Midi Kits were used to extract RNA from the cells following the manufacturer's instructions. Briefly, the cell pellet was re-suspended in a volume of buffer RLT (containing β-Mercaptoethanol at 0.01ml per 1ml buffer RLT) depending on the number of cells harvested; $5x10^6$–$3x10^7$ cells: 2ml; $3x10^7$–$5x10^7$ cells: 2ml; and $5x10^7$–$1x10^8$ cells: 4ml.

The lysate was passed through an 18-20 gauge needle to homogenise the cells, an equal volume of 70% ethanol added and the sample vigorously shaken. The sample was applied to the midi column and centrifuged at 5000rpm for 10 minutes. A series of wash steps was then completed, the column placed in a new collection tube and 70µl of RNase/DNase-free water was pipetted onto the membrane. The column was incubated at room temperature for 1 minute and centrifuged. The elution was then transferred from the collection tube back to the membrane and centrifugation repeated. The quality and quantity of the RNA was assessed before being stored at -80°C.

RNA precipitation procedure

When the concentration of RNA obtained from an extraction was not sufficient for further work, the RNA was precipitated as a pellet and re-suspended in a smaller volume of DEPC-treated water. Initially, a tenth of the sample volume of 3M sodium acetate (NaOAc) pH 5.2 was added to the RNA sample, followed by two and a half times the sample volume of absolute ethanol. The sample was then gently vortexed. Prior to incubation overnight at -20°C, 5µg of glycogen was added to increase the sample pellet visibility. Once incubated, the sample was centrifuged at 15,000rpm for 20 minutes, the resulting pellet washed in 80% ethanol and centrifuged at 10,000rpm for 7 minutes. This step was repeated. The ethanol was then removed and the pellet air-

dried. Finally the pellet was re-suspended in the desired volume of DEPC-treated water and stored at -80°C.

DNA

DNA extraction from biopsy tissue

All DNA extractions were completed in a sterile Astecair 5000E hood (Bioquell UK Ltd). Approximately 25mg of tissue was used for each extraction depending on the initial tissue size.

DNA was extracted from the biopsy tissue using the QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's instructions. All buffers were supplied with the kit. Briefly, 25mg of tissue was diced and placed in a 1.5ml microcentrifuge tube containing 180µl of buffer ATL and 20µl of proteinase K (0.6AU/ml). The sample was vortexed and incubated at 56°C for 3 hours or until the tissue was completely lysed. To remove any RNA contamination, 4µl of RNase A (100mg/ml) was then added to the sample followed by 200µl of Buffer AL. The sample was pulse-vortexed and incubated at 70°C for 10 minutes. 200µl of ethanol (96%-100%) was added, the mixture transferred to the QIAamp Spin Column and centrifuged at 800rpm for 1 minute. A series of wash steps was then completed, the column placed in a new collection tube and 200µl of DEPC-treated water added to the column. The column was centrifuged at 800rpm for 1 minute to elute the DNA. DNA quality and quantity was assessed before being stored at -80°C.

DNA extraction from cell cultures

Initially, cells that were 70% confluent in three 150cm^3 flasks were detached using trypsin and counted using the Coulter Counter as previously described. Qiagen Genomic-tips were then used to extract DNA from the cells once they had been homogenised. All buffer preparations can be found in Appendix I.

Cells were resuspended in 2ml of ice cold PBS (1x phosphate buffered saline) at x10^7 cells per ml. An equal volume of ice cold buffer C1 was added to the suspension, the tube inverted several times and incubated on ice for 10 minutes. The sample was centrifuged at 4°C for 15 minutes at 900rpm to pellet the cells. The supernatant was removed and the pellet resuspended in 1ml ice cold buffer C1 and 3ml of ice cold

DEPC-treated water. The sample was then vortexed and centrifuged as previously described. The supernatant was discarded, 5ml of buffer G2 was used to resuspend the pellet and the sample was vortexed for 10-30 seconds. To digest any denatured proteins, 95µl of protease (1AU/ml) was added to the sample which was then incubated at 50°C for 45 minutes or until the lysate became clear.

Gravity allowed all solutions to pass through the genomic-tip 100/G. Initially, the tip was equilibriated with 4ml of buffer QBT. Once vortexed for 10 seconds, the lysate was applied to the tip, this was followed by a wash step using 7.5ml of buffer QC. The genomic DNA was then eluted by applying 5ml of buffer QF to the tip. DNA precipitation was achieved by adding 3.5ml of room temperature isopropanol to the elution, vortexing briefly and centrifuging at 4°C for 15 minutes at max speed. The supernatant was removed leaving the DNA pellet which was then washed in 2ml of ice cold 70% ethanol and centrifuged at 4°C for 10 minutes at max speed. The ethanol was removed and the pellet allowed to air dry before resuspension in 100µl of DEPC-treated water. Finally, the DNA was dissolved overnight at 55°C. DNA quality and quantity was assessed before being stored at -80°C.

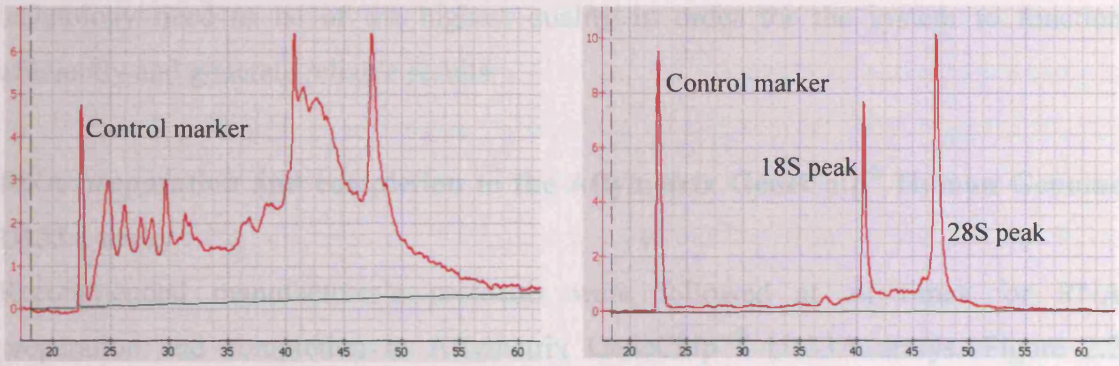
RNA and DNA quantification and assessment

Agilent Bioanalyser 2100

The Agilent Bioanalyser 2100 and RNA 6000 NanoChip® assay (Agilent Technologies) were used to assess RNA quality and quantity. High quality total RNA samples were characterised by an electropherogram with two well-defined peaks corresponding to 18S and 28S ribosomal RNAs, with peak ratios between 1.9 and 2.1. A lower ratio and distorted peaks indicates RNA degradation.

The priming station and initial RNA 6000 NanoChip® preparations were carried out according to manufacturer's instructions. For sample analysis, 5µl of RNA 6000 Nano marker and 1µl of sample were added to each well. The chip was vortexed for 1 minute and inserted into the Bioanalyser. The running of the chip was controlled through the Agilent Bioanalyser 2100 software.

Figure 2.1 The eletropherograms illustrate degraded RNA (a) and high quality RNA (b).



(a) (b)

Spectrophotometer analysis

DNA and RNA quality and quantity was also assessed using a spectrophotometer (GeneQuant, Pharmacia Biotech, Amersham Bioscience). An absorbance measurement at 260nm of 1 is equivalent to 40μg/ml of RNA or 50μg/ml of DNA and these values were used to calculate RNA or DNA concentrations. An additional measurement at 280nm was also taken and the ratio (260nm/280nm) calculated. Good quality RNA and DNA should have a ratios between 1.9 and 2.1 or 1.7 and 1.9 respectively. A value lower indicates nucleotide degradation and a value higher indicates protein contamination.

Affymetrix GeneChip® Human Genome U133A array

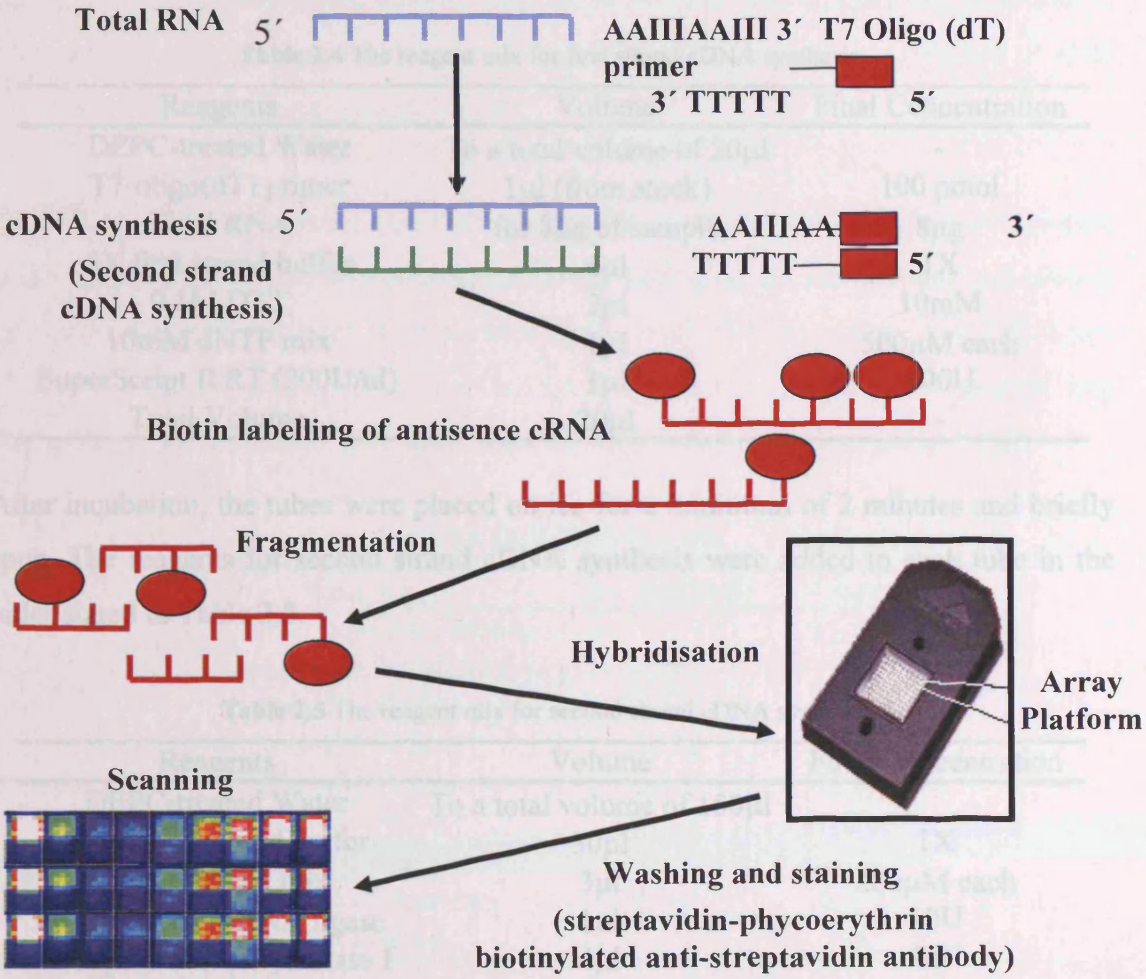
The Affymetrix GeneChip® U133A array allows the analysis of genome-wide gene expression in a single experiment. The array encompasses 22000 probes sets including 18400 transcripts and variants that correlate with 14500 well-characterised genes. The array platform is divided into a series of 'spots'. Oligonucleotide probe pairs complementary to corresponding gene sequences are synthesised *in situ* to each spot. The probe pairs are 25 bases in length and designed to be a match and a mismatch to the target cRNA. On every array, each probe set consists of 11-16 oligonucleotide probe pairs. This multi-detection method increases the sensitivity and specificity of transcript detection in the presence of complex background signals. This is particularly important when looking at low abundance genes. The gene sequences used in the array design were selected from GeneBank®, dbEST and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and then refined by analysis and

comparison with other public databases. Samples used for Affymetrix GeneChip® array technology need to be of the highest quality in order for the system to function efficiently and generate reliable results.

RNA preparation and completion to the Affymetrix GeneChip® Human Genome U133A array

Recommended manufacturer's protocols were followed at all times for RNA preparation and completion to Affymetrix GeneChip ® U133A arrays. Figure 2.2 illustrates each stage of the protocol.

Figure 2.2 cRNA synthesis from total RNA for microarray analysis.



cDNA synthesis from total RNA

Initially, 8μg of total RNA was required for cDNA synthesis. Table 2.4 indicates the reagents, volumes and concentrations used for one cDNA synthesis reaction per sample. All reagents were purchased as the Superscript Choice System (Invitrogen Ltd). To initiate first strand cDNA synthesis, DEPC-treated water, T7-oligo (dT) primer and total RNA were added to a 0.5ml tube. Each tube was flick mixed, briefly spun and incubated at 70°C for 10 minutes. To complete the primer hybridisation, the tubes were placed on ice for a minimum of 2 minutes. The 5X first strand buffer, DTT and dNTP mix were added, the tubes flick mixed, briefly spun and incubated at 42°C for 2 minutes. Superscript II was then added, the tubes mixed, spun and incubated at 42°C for 1 hour (Table 2.4).

Table 2.4 The reagent mix for first strand cDNA synthesis.

Reagents	Volume	Final Concentration
DEPC-treated Water	To a total volume of 20μl	-
T7-oligo(dT) primer	1μl (from stock)	100 pmol
Total RNA	for 8μg of sample	8μg
5X first strand buffer	4μl	1X
0.1M DTT	2μl	10mM
10mM dNTP mix	1μl	500μM each
SuperScript II RT (200U/ul)	1μl	200U
Total Volume	20μl	-

After incubation, the tubes were placed on ice for a minimum of 2 minutes and briefly spun. The reagents for second strand cDNA synthesis were added to each tube in the order stated in Table 2.5.

Table 2.5 The reagent mix for second strand cDNA synthesis.

Reagents	Volume	Final Concentration
DEPC-treated Water	To a total volume of 150μl	
5X second strand buffer	30μl	1X
10mM dNTP mix	3μl	200μM each
10U/μl *E.Coli* DNA ligase	1μl	10U
10U/μl *E.Coli* polymerase I	4μl	40U
2U/μl *E.Coli* RNase H	1μl	2U
Total Volume	150μl	

The tubes were flick mixed, briefly spun and incubated at 16°C for 2 hours. The tubes were then spun, 2μl of T4 DNA polymerase (10U) added and the tubes incubated at

16°C for a further 5 minutes. Finally, 10µl of EDTA (0.5M) was added to each tube and the samples cleaned.

Clean-up of double-stranded cDNA

cDNA clean-up was completed using the Sample Clean-up Module (Affymetrix Inc.) according to the manufacturer's instructions. To each sample, 0.6ml of cDNA binding buffer was added and vortexed for 3 seconds. The colour of the mixture was always yellow indicating correct pH. The sample was applied to the cDNA clean-up column and centrifuged at 10,000rpm for 1 minute. A wash step was then completed and the column centrifuged with the cap open at maximum speed for 5 minutes to dry the membrane. The columns were transferred to a new 1.5ml collection tube and 14µl of cDNA elution buffer pipetted directly onto the membrane. The column was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute to elute the cDNA. The cDNA was stored at -20°C.

To ensure cDNA synthesis had been successful, a reverse transcriptase polymerase chain reaction (RT-PCR) was completed for the gene β-actin. The forward and reverse primer sequences used are as follows; forward: 5'-AATCTGGCACCACACCTTCTAC-3' and reverse: 5'-CTCCTTAATGTCACGCACGAT-3'. The RT-PCR reaction mix was made up as shown in Table 2.6 and the cDNA synthesis reaction diluted 1:20.

Table 2.6 RT-PCR reaction mix.

Reagent	Concentration	Volume
RNase/DNase Free Water	-	13.8µl
Buffer Without $MgCL_2$	10x	2µl
$MgCL_2$ Buffer	25mM	1.2µl
dNTP Mix	10mM	0.5µl
Forward Primer	10mM	0.5µl
Reverse Primer	10mM	0.5µl
cDNA Template	(1:20)	1µl
Taq DNA Polymerase	5U/µl	0.5µl
Total Volume	-	20µl

The reaction cycle parameters, included an initial denaturation step at 94°C for 2 minutes, followed by denaturation phase at 94°C for 30 seconds, an annealing phase at 60°C for 30 seconds and extension phase of 72°C for 45 seconds. This was repeated 30 times followed by a final step of 72 °C for 4 minutes. Once the reaction was completed

the tubes were placed on ice for a minimum of 2 minutes and spun to collect any condensation.

Gel electrophoresis was performed to view the amplified β-actin product. A 3% agarose gel was made using 60ml of 1X tris-acetate (TAE) buffer and ethidium bromide (10μg/ml). 2μl of DNA loading-dye was added to each 20μl cDNA sample reaction mix and a 100bp DNA ladder (MBI Fermentas) was used as a marker for the 350bp β-actin product. Products were then visualised using a UV transilluminator. A previously successful cDNA sample was included in this RT-PCR reaction as a positive control.

Biotin labelled cRNA synthesis

The Bio Array High Yield RNA Transcript Labelling Kit (Affymetrix Inc.) was used to generate biotin-labelled cRNA using 10μl of the cDNA synthesis reaction. The reaction for each sample was set up as shown in Table 2.7. The tubes were flick mixed, spun and incubated at 37°C for 5 hours. At 30 minute intervals, the samples were gently mixed via pipetting. Once complete, the samples were placed on ice for a minimum of 2 minutes and cleaned.

Table 2.7 Reagent mix for cRNA synthesis reaction.

Reagent	Volume
Template cDNA	10μl
RNase/DNase Free Water	12μl
10X HY Reaction Buffer	4μl
10X Biotin-Labelled Ribonucleotides	4μl
10X DTT	4μl
10X RNase Inhibitor Mix	4μl
20X T7 RNA Polymerase	2μl
Total Volume	40μl

Clean-up of biotin labelled cRNA

Buffers were made in the laboratory according to the manufacturer's instructions (Sample Clean-up Module, Affymetrix Inc). Initially, 60μl of RNase/DNase free water, 350μl of IVT cRNA binding buffer and 250μl of absolute ethanol were added to each cRNA reaction and vortexed for 3 seconds. The mixture was then transferred to an IVT cRNA clean-up column and centrifuged at 10,000rpm for 15 seconds. A wash step was then completed followed by a second involving 0.5ml of 80% ethanol. The column was

then centrifuged with the cap open at max speed for 5 minutes to dry the membrane. The columns were then transferred to a new 1.5ml collection tube and 11µl of RNase/DNase free water pipetted directly onto the membrane. The column was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute. This step was repeated with 10µl of RNase/DNase free water. cRNA quality and quantity was assessed by spectrophotometer and gel electrophoresis analysis. The samples were then stored at -80°C.

Correction calculation

The yield of cRNA was adjusted to reflect the carry over of unlabelled total RNA. The spectrophotometer analysis result was corrected using the following calculation.

$$\text{Adjusted cRNA yield} = RNA_m\text{-(total } RNA_i\text{) (y)}$$

RNA_m = amount of cRNA measured after IVT (µg)

total RNA_i = starting amount of total RNA (µg)

y = fraction of cDNA reaction used in IVT

Fragmentation of biotin labelled cRNA

The fragmentation mix shown in Table 2.8 was used for each sample followed by an incubation step at 94°C for 35 minutes. The samples were placed directly on ice for a minimum of 2 minutes and briefly spun to collect any condensation. To ensure fragmentation had been successful, 2µl of mixture was removed and assessed via gel electrophoresis. The samples were then stored at -20°C.

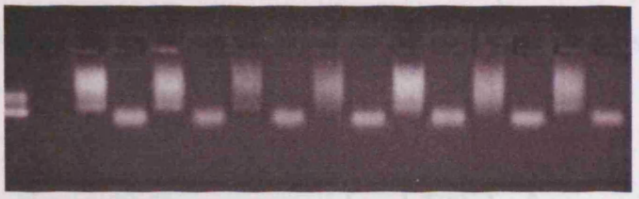
Table 2.8 The reaction mix required for fragmentation.

Component	Volume
15µg cRNA	Sufficient for 15µg
5X Fragmentation Buffer	8µl
RNase/DNase Free Water	To a total volume of 40µl

cRNA assessment

Gel electrophoresis was used to asses the quality of both the cleaned cRNA and the fragmented cRNA. This was carried out as previously described using a 1% agarose gel and a 1kb RNA Century Marker (Ambion Ltd) (Figure 2.3).

Figure 2.3 The gel electrophoresis image illustrates cleaned and fragment cRNA.



The 1kb marker was located in the first well (the second well is empty) and the samples IN3013, IN2044, IN2003, IN2591, IN2102, IN1751 and IN2921 are located in wells 3-16; cleaned cRNA followed by fragmented cRNA for each sample.

cRNA hybridisation

The hybridisation cocktail assembled for each fragmented cRNA sample was composed of the reagents shown in Table 2.9 including the GeneChip® Eukaryotic Hybridisation Control Kit (Affymetrix Inc.), Herring Sperm (Invitrogen) and BSA (Promega). The samples were flick mixed, briefly spun and incubated at 99°C for 5 minutes. The samples were the transferred to 45°C for 5 minutes before being spun at maximum speed for 5 minutes.

Table 2.9 Reaction mix required for each hybridisation cocktail.

Component	Volume
15µg Fragmented cRNA	sufficient for 15µg
Control Oligonucleotide B2 3nM	5µl
20X Eukaryotic Hybridisation Controls (bioB, bioC, bioD, cre)	15µl
Herring Sperm DNA (10mg/ml)	3µl
Bovine Serum Albumin (BSA) (50mg/ml)	3µl
2X Hybridisation Buffer	150µl
DMSO	15µl
RNase/DNase Free Water	to final volume 300µl
Total Volume	300µl

The Affymetrix GeneChip® U133A probe array was allowed to reach room temperature before use. The array was primed using 200µl of 1X hybridisation buffer via the septa on the probe array reverse and incubated at 45°C for 10 minutes. The hybridisation

buffer was then removed and 200µl of the treated hybridisation cocktail pipetted into the probe array. The array was then placed into the hybridisation oven at 45°C for 16 hours.

U133A probe array washing and staining

The fluidics Station 450 was used to wash and stain the probe arrays. This was set up and primed according to Affymetrix protocol guidelines prior to this stage of the array protocol. All buffer synthesis protocols can be found in Appendix I.

After hybridisation, the cocktail was removed and 200µl of non-stringent wash buffer was added to the probe array. The array was inserted into the primed fluidics station and the EukGE-WS2v4 _450 washing and staining protocol carried out (Table 2.10).

Table 2.10 Washing and staining protocol for Affymetrix GeneChip®.

	EukGE-WS2v4 _450 wash protocol
Post Hybridisation Wash #1	10 cycles of 2 mixes/cycle with wash buffer A at 25°C
Post Hybridisation Wash #2	4 cycles of 15 mixes/cycle with wash buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	10 cycles of 4 mixes/cycle with wash buffer A at 25°C
2nd Stain	Stain the probe array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	15 cycles of 4 mixes/cycle with wash buffer A at 30°C. The holding temperature is 25°C.

Wash Buffer A = Non-Stringent Wash Buffer; Wash Buffer B = Stringent Wash Buffer

U133A probe array scanning

Once the wash and staining protocol was complete the probe arrays were scanned using the Affymetrix GeneChip® Scanner 3000 following manufacturer's guidelines. Before the probe array was inserted into the scanner, Tough-Spots™ were placed over the two septa to prevent fluids leaking during scanning. The array was then scanned at a pixel resolution of 3µm and wavelength of 570nm.

The raw data the intensities of each probe set collected by the scanner dictated the expression measurement. This measurement is described both qualitatively and quantitatively and compared to a baseline line measurement to gauge the relative change in transcript abundance. Using the probe pair intensities and a detection algorithm, a detection p-value is generated that indicates the reliability of transcript detection. This can be either present, marginal or absent. A further algorithm uses the probe pair intensities to calculate a signal value which assigns a relative measure of abundance of each transcript. These expression results can then be analysed using a variety of software programmes.

GeneSpring® - Affymetrix GeneChip® Human Genome U133A array data analysis

GeneSpring® allows the analysis of all 22000 gene expression results generated by the Affymetrix GeneChip® Human Genome U133A array. Samples were grouped into specific experiments to answer the different questions laid out in the aims of this project. Only 1 array chip was completed per sample and therefore the cross-genes error model was applied when the samples were not grouped according to a common parameter. The cross-gene error model assesses sample and measurement variation as a function of the control strength within all the measurements taken for a single sample, compared to the usual method of sample replicates to assess variation. This is achieved by ordering the probe sets by their control strengths and calculating the median variance and median control strength for each non-overlapping set of eleven. The error for each probe set is approximated by using the median deviation from 1.0. This approach can be used with general purpose arrays where the majority of genes have little biological variability.

Normalizations were then carried out to standardize all data in an experiment. This reduces the affects of background noise and experimental variation enabling biological variation in gene expression to be more easily identified. This also allows data scaling so that relative gene expression comparisons can be made between samples. Initially, a data transformation normalisation was completed for an experiment. Any measurements less than 0.01 (set as a standard cut-off) were readjusted to 0.01. This removed any negative values given as a gene expression level and replaced it with a level equivalent to 0 (i.e. no expression). Per chip normalisations were then completed, dividing all the

measurements on each array by the 50th percentile. This corrected for chip-wide variations in intensity, possibly due to inconsistent washing, sample preparation or array production. The resulting expression levels were therefore centred on 1 for each chip. Measurements for this normalisation step were limited to those that were called as present or marginal.

Finally, per gene normalisations were completed. In this approach, the expression level of each gene was divided by either the mean expression of the same gene in several control samples or the mean expression of the gene in all samples. Normalisations were carried out in sequence using the corrected gene values from the previous normalization. The choice of normal control samples is most important when using this approach to normalise an experiment. The reliability and number of control samples was considered when experiment analysis was completed.

Once the raw expression data for a given experiment had been normalised, various filters were applied to remove unwanted or unnecessary results from the final stage of analysis. Using the tool, Filter On Flags, gene expression results were removed from the experiment according to detection reliability on the chip; absent, marginal or present. Expression results flagged absent in every sample were removed from an experiment. Removal of further gene expression data depended on the number of present calls per number of samples in the experiment. Genes that were identified as being constant in all samples and Affymetrix GeneChip® standard genes were also removed from further analysis.

Allowing genes that are present in a proportion of samples, for example 50%, into an experiment permits those genes that are not expressed due to sample biology rather than an experimental error to be analysed. Moreover, this allows those genes that are not expressed in a control group compared to a sample group or not expressed in a sample group compared to a control group to be investigated. This approach does allow more false positives into an experiment but at the same time provides a more accurate assessment of gene expression profiles with regard to genes that are expressed and not expressed. Few microarray studies using GeneSpring® software for analysis state the 'present' criteria included in the study. However, one study stated that 'the magnitude

of difference between two groups was defined using the following criteria; (i) greater than 2-fold difference, (ii) statistical significance ($p < 0.05$), and (iii) classification as 'present' in at least one of the tumours' (Sowar et al., 2006). Furthermore, Wong et al. (2005) removed genes that were called 'absent' in 5 of the 21 tumours investigated.

From the final group of reliable gene expression results, those genes that showed a statistically significant difference between groups were identified. This was completed using a Student's t-test or a one-way ANOVA and variances were assumed to be equal. A p-value cut-off of $p = 0.05$ was used in each test. In order to reduce the false discovery rate, the Benjamini and Hochberg false discovery rate multiple test correction was also applied. In addition, the tool Filter On Fold was used to find those genes that showed a greater than 2-fold up or down-regulated between groups. Genes identified by both methods were used in further analysis approaches. When investigating one sample, statistical analysis was not always possible. Consequently, a gene fold change greater than 2 was used to identify differentially expressed genes. The functional significance of those genes deemed to be differentially expressed between groups could only be determined by further analysis approaches discussed below. The expression fold change and level of significance limits used in this study reduced the overall gene list to a manageable number for these approaches. Furthermore, genes outside these limits may also show differential expression and play a biological role in astrocytoma development or progression. The separation of genes according to a 2-fold change in expression between groups may exclude some genes with biological significance. However, this fold change was chosen as it was felt that fewer genes below this cut-off would have functional significance compared to higher changes of 3 or 4-fold often used. Consequently, all genes within pathways of interest that were identified using this fold change and statistical limits, were investigated for changes in gene expression.

Unsupervised hierarchical clustering approaches were used to cluster the samples and controls according to expression profile similarity. This method establishes samples with similar gene expression patterns, highlighting groups and sub-groups regardless of any sample or patient parameter. Separation ratios determine the similarity between two samples or groups of samples, with a ratio of 0 indicating identical expression profiles. This approach allows sample and patient parameters to be correlated with sample gene expression patterns (Eisen et al., 1998). This clustering approach was also used to

cluster the genes according to expression profile similarity and where appropriate k-means clustering was used to identify sets of genes with similar expression patterns.

K-means clustering groups genes into sets that show a high degree of expression similarity within each set but a low degree of similarity between sets. The clustering algorithm divides genes into a user-defined number of groups. To do this, centroids (in expression space) are created at the average location of each set of genes and each algorithm iteration assigns genes to the set with the closest centroid. After each iteration, the location of the centroid is recalculated and the process repeated. This results in the average expression profile for each set being distinct from that of others (Shai et al., 2003; Datta and Datta, 2006; Steinley, 2006).

Aberrantly expressed genes were investigated in detail using the internet based software Onto-tools (Draghici et al., 2003; Khatri et al., 2005). This software generated functional profiles of the differentially expressed genes and assessed the impact of alterations upon biological processes and pathways. The pathways used by Onto-tools originate from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). A list of the pathways involved in this approach can be found on the Supplementary Data CD included with this thesis. Gene functions, pathway involvements, chromosomal locations and links with other tumours or cancers were the main factors studied.

Q-PCR

To validate the Affymetrix GeneChip® U133A array results, Q-PCR analysis was completed for a number of genes using QuantiTect Reverse Transcription Kit (Qiagen). All buffers were supplied with the kit and a genomic DNA elimination step was completed for each sample prior to Q-PCR. cDNA synthesis was completed using 1μg of total RNA according to manufacturer's instructions. A reverse transcription master mix was prepared for each sample using the kit components, which included an RT buffer, an RT primer mix and reverse transcriptase. The master mix was divided into individual reactions, the genomic DNA-free total RNA for each sample was added to each reaction, the mixture flick mixed, briefly centrifuged and incubated at 42°C for 15 minutes. To inhibit the reaction, the mixture was incubated for 3 minutes at 95°C. The

cDNA was then diluted 1:10 using DEPC-treated water and 1µl of the resulting mixture was used for Q-PCR.

Q-PCR was carried out using Assays-on-Demand™ (Applied Biosystems). Gene-specific oligonucleotide probes and primer pairs for the genes of this study can be found in Appendix I. All probes were designed across exon-exon boundaries. Taqman® Universal Mix No AmpErase UNG was used in a 25µl reaction volume following cycling conditions recommended by the manufacturer. These involved an initial denaturation step of 10 minutes for 95°C followed by forty cycles of 95°C for 15 seconds and 60°C for 1 minute.

All reactions were completed as single well reactions using the ABI prism 7000 Sequence Detection System. The comparative C_T method (PE Applied Biosystems) was used to determine the relative ratio of the gene expression for each gene in the tumour samples compared to the normal controls. *β-actin* was used as an endogenous control.

$$\frac{X_{test}}{X_{control}} = 2^{-\Delta\Delta C_T} = 2^{-(C_{T,X}-C_{T,R})_{control}-(C_{T,X}-C_{T,R})_{test}}$$

$C_{T,X}$ is the cycle threshold of the gene of interest and $C_{T,R}$ is the cycle threshold of the endogenous control. Test, refers to the tumour cDNA sample and Control, refers to the normal brain cDNA control sample.

Methylation specific PCR assay and sequencing

DNA bisulfite treatment

All buffer preparations can be found in Appendix I.

DNA was treated with sodium bisulfite to convert all unmethylated cytosines to uracil as described previously (Herman et al., 1996). Briefly, 1µg of genomic DNA was diluted in 40µl of RNase/DNase free water, 10µl of NaOH added, the sample flick mixed, briefly centrifuged and incubated at 37°C for 10 minutes. To this mixture, 30µl of 10mM hydroquinone and 520µl of 3M sodium bisulfite were added, the tube inverted, briefly centrifuged and mineral oil (Sigma) layered over the contents. The sample was then incubated at 50°C for 16-20 hours. Once incubated the sample was

transferred from beneath the oil to a new eppendorf tube and cleaned using the Wizard® DNA Clean-Up System (Promega) following manufacturer's instructions.

Initially, a 3ml disposable syringe was attached to the Wizard® DNA mini column. 1ml of Wizard® DNA clean-up resin was added to each sample, the sample inverted and the slurry added to the syringe barrel of the Wizard® column. Pressure was applied to move the slurry through the column. A wash step was completed using 2ml of 80% isopropanol, the syringe was removed and the column transferred to a 1.5ml eppendorf tube. The column was centrifuged at maximum speed for 2 minutes to dry the membrane. To elute the DNA, 30µl of warmed DEPC-treated water was added to the membrane, the column incubated for 1 minute at room temperature and centrifuged at maximum speed for 20 seconds. This step was repeated with 25µl of warmed DEPC-treated water.

To pellet the DNA, 6.1µl of 3M NaOH was added to each elution, the mixture flick mixed and incubated at room temperature for 5 minutes. Two volumes of ice cold absolute ethanol were added to the sample, the tube inverted and incubated on ice for 1 hour. The sample was then centrifuged at 13,000rpm for 15 minutes. The supernatant was removed, 300µl of ice-cold ethanol added to the visible pellet, the sample centrifuged as previously described and the step repeated. The supernatant was then removed and the pellet allowed to air dry. The DNA was resuspended in 25µl of TE buffer and stored at -20 °C.

Methylation specific PCR

The methylation specific PCR (MSP) and sequencing primer pairs for this study were either adopted or designed according to MSP principles (Olek et al., 1996a; Warnecke et al., 1997) with the aid of the CpG island identification web server (http://www.uscnorris.com/cpgislands2/), primer design web server and software packages (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Primer Express (Table 2.11). A blank control containing all MS-PCR components except sample DNA and two positive controls (normal DNA and commercially purchased universally methylated DNA (CpGenome™) both of which were bisulphite-treated) were included in each experiment.

Table 2.11 MSP primer sequences, annealing temperatures and product sizes for all genes investigated.

Genes	Sense	Annealing Temperature (°C)	PCR Product Size
CCNA1	Sense (M) tcgtcgcgttttagtcgt	54.3	200
	Sense (U) gggtagttttgttgtgttttagtt		
	Antisense (M) cgttctcccaacaaccg	55.1	200
	Antisense (U) ccattctcccaacaaccac		
CDKN1C	Sense (M) gggttcgcgcgtataaa	54.7	200
	Sense (U) gggtggggtttgtgtgtat		
	Antisense (M) atacgaaaaacgcgacgac	53.7	200
	Antisense (U) aaaacacaacaactacctaactatcc		
DAPK1	Sense (M) gtcggatcgagttaacgtcg	56.9	70
	Sense (U) gtttgtgagtgagtgtttagtttg		
	Antisense (M) aaacgccgaccccaaa	57.6	70
	Antisense (U) tctaattacctaaaaccaaattcatca		
PRDM2	Sense (M) cgacggcgtagggttaagg	58.7	150
	Sense (U) ggtgatggtgtagggttaagggt		
	Antisense (M) ctatttcgccgaccccg	58.7	150
	Antisense (U) cactatttcaccaaccccaaca		
REPRIMO	Sense (M) tgcgagtgagcgtttagttc	55.1	100
	Sense (U) gtttgtgagtgagtgtttagttgg		
	Antisense (M) ttacctaaaaccgaattcatc	55.7	100
	Antisense (U) tctaattacctaaaaccaaattcatca		
SPINT2	Sense (M) cgagaaggtcgggcg	55.7	150
	Sense (U) gttgagaaggttgggtgtttt		
	Antisense (M) caaccgttaaaatctcgcg	54.3	150
	Antisense (U) caccaaccattaaaatctcac		

MSP was carried out in a 25µl volume (Table 2.12) with Qiagen Hot Start Taq using 1.5µl of bisulfite treated DNA. The initial denaturation step at 95°C for 15 minutes was followed by an annealing step for 1 minute at the desired primer pair temperature. Thirty five amplification cycles were completed at 95°C for 30 seconds, varying primer pair temperatures for 30 seconds and 72°C for 30 seconds. A final extension step at 72°C for 5 minutes was then completed.

Table 2.12 Components, concentrations and volumes of the MSP reaction.

Component	Concentration
Modified DNA Sample	50-100ng
MSP Buffer	10X
12.5mM dNTP	0.25mM
10µM Forward Primer	0.2µM
10µM Reverse Primer	0.2µM
Hot Start Taq	1U
DEPC-treated water	-

Polyacrylamide gel electrophoresis was used to separate MSP products due to their small size. A 10ml gel mix consisting of 10% glycerol was prepared using 2ml of 29:1 Accugel and 1ml of 10X TBE. To initiate polymerisation, 100µl of 10% APS

(ammonium sulphate) and 10µl of TEMED were added to the glycerol mix. This was allowed to polymerise at room temperature for 20 minutes. Electrophoresis was carried out in 1X TBE at 90V for 1.5 hours. The gel was incubated in 50ml of 1X TBE containing ethidium bromide (0.5µg/ml), for approximately 20mins. The products were visualised by UV light.

Sequencing of bisulfite treated DNA

PCR for sequencing was carried out in a 50µl volume with Qiagen Hot Start Taq using the primers in Table 2.13. After an initial incubation at 95°C for 15 minutes, 38 cycles of 94°C for 15 seconds, varying primer temperatures for 30 seconds and 72°C for 45 seconds were completed. This was followed by a final extension step at 72°C for 5 minutes. Products were separated using a 1.5% gel and TBE buffer. The products were visualised by UV light.

Table 2.13 Sequencing primers, annealing temperatures and product sizes for all genes studied.

Genes	Sense	Annealing Temperature (°C)	PCR Product Size
CCNA1	Sense (Set1) ggggaagatttttgtggg Antisense (Set1) tcaaacccctaaaaacccaac	56	525
	Sense (Set2) tatagttggagttggagggt Antisense (Set2) caaaacctacctaaacccc	54	490
CDKN1C	Sense (Set1) gtggtgttgttgaaattga Antisense (Set1) cactaatactaaaaaaatcccac	49	420
	Sense (Set2) gtgggatttttttagtattagtg Antisense (Set2) accaaaacactaaacaactactc	49	375
DAPK1	Sense (Set1) ggaaggtagggatttaaaaa Antisense (Set1) accaataaaaaccctacaaac	49	380
	Sense (Set2) gtttgtagggtttttattggt Antisense (Set2) ccttaaccttcccaattact	49	450
	Sense (Set3) gagtaattgggaaggttaagg Antisense (Set3) tcactaaaaacaatctctctcca	52.5	380
PRDM2	Sense (Set1) tgggaatagtaagttttttaaggg Antisense (Set1) caataaccaccacccaacc	55	420
	Sense (Set2) ggttgggtggtggttatt Antisense (Set2) aaaaccccaaataacccca	55	600
REPRIMO	Sense (Set1) gggtttgtttttggttaatagtag Antisense (Set1) caaatttacttcacccaatctc	52.5	390
SPINT2	Sense (Set1) aggaaggaatttataggaaagttg Antisense (Set1) ccaaataccaaaaccccc	53.5	575
	Sense (Set2) gggggttttggtatttgg Antisense (Set2) attctccctactcaaacccc	57	320

The products were excised from the gel and cleaned for sequencing using the PureLink™ Quick Gel Extraction Kit (Invitrogen) following manufacturer's instructions. Excised bands were placed in a 1.5ml eppendorf tube and weighed. For

every 10mg of gel, 30µl of stabilisation buffer was added and the solution incubated at 50°C for 15 minutes. The mixture was vortexed every 3 minutes to promote gel dissolution. The mixture was loaded into a Quick Gel Extraction column and centrifuged at 12,000rpm for 1 minute. A series of wash steps were then completed. To elute the DNA, 50µl of warmed TE buffer was added to the membrane and the column incubated at room temperature for 1 minute. The column was then centrifuged at 12000rpm for 2 minutes.

Individual sequencing reactions using both forward and reverse primers was completed for each gene in 2 tumour samples and 1 normal control. The reaction was carried out using the sequencing PR-100 buffer and 5X reaction buffer from Applied Biosystems in a 20 µl total volume following manufacturer's guidelines. An initial denaturation step was completed at 96°C for 30 seconds. This was followed by 25 cycles at 50°C for 15 seconds and 60°C for 4 minutes.

The sequencing reaction was cleaned using the Millipore Montage™ Single Sample PCR Kit (Montage). Briefly, 380µl of DEPC-treated water and the sequencing reaction mixture was added to the Millipore column and centrifuged at 1,000rpm for 15 minutes. To elute the sequencing product, the column was turned upside-down, placed into a clean eppendorf tube, 20µl of TE buffer added to the membrane and the column centrifuged at 1,000rpm for 2 minutes. Finally, 10µl of the sequencing product was transferred to a 96 well plate and sequence analysis completed by the 3730 DNA Analyser. Raw data analysis and sequence viewing was completed using the ABI SeqScape software.

SpectralChip™ 2600 array

The SpectralChip™ 2600 is a human BAC array that generates a molecular profile of each chromosome allowing quantitative analysis. Each 2600-element BAC array is printed on a glass slide and evaluates genome wide regions of gain and loss at a resolution of 1Mb.

DNA preparation and completion to the SpectralChip™ 2600 array

The SpectralChip™ 2600, Spectral hybridisation buffers and Cy5-dCTP and Cy3-dCTP dyes were purchased from GE Healthcare. The male and female reference DNAs were

purchased commercially and are composed of multiple anonymous donors according to the manufacturer's description (Promega). All additional buffers for this protocol were made in the laboratory and can be found in Appendix I.

Differential labelling of DNA with Cy3-dCTP and Cy5-dCTP

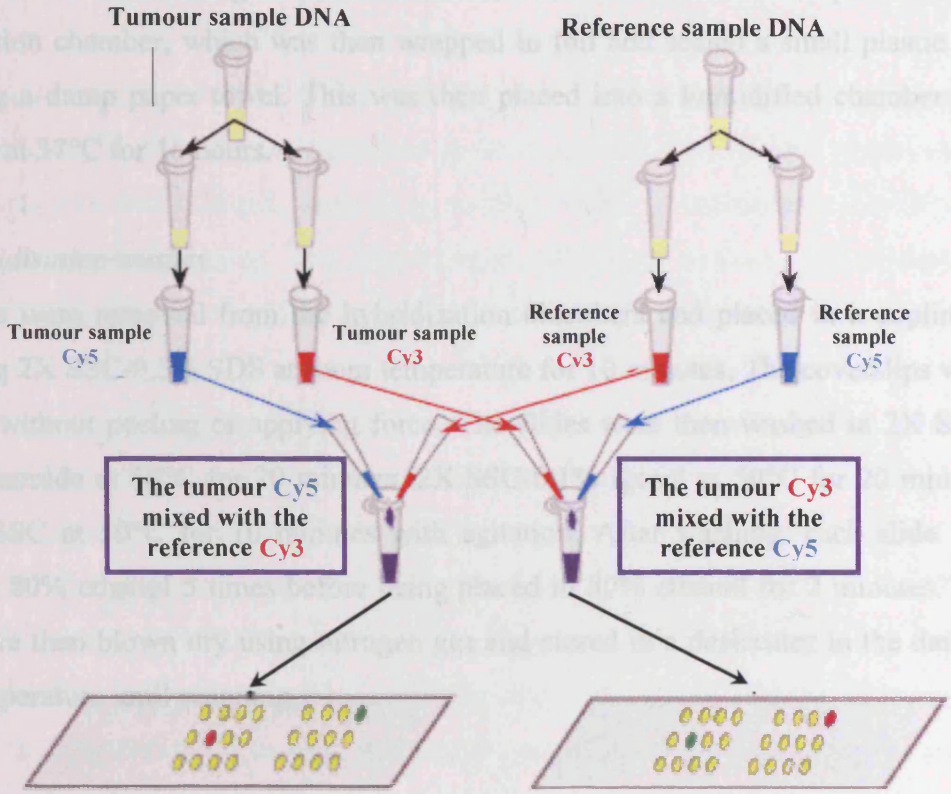
Initially, 1μg of tumour DNA and 1μg of reference DNA were each made up to a volume of 25μl using DEPC-treated water. To each sample, 25μl of 2.5X random primer solution (Bioprime Labelling Kit, Invitrogen) was added and the samples heated at 100°C for 5 minutes. The samples were then placed into an ice slurry for 5 minutes and briefly centrifuged. A dye master mix including labelling buffer, klenow fragment and either Cy5-dCTP or Cy3-dCTP was made up following manufacturer's instructions, according to the number of samples being processed. The dye was kept in the dark at all times and was not used in direct sunlight or artificial lighting.

Once the master mix had been made, 5μl of the Cy5 dye mix was added to the tumour sample and 5μl of the Cy3 dye mix was added to the corresponding reference sample. The samples were then flick mixed, briefly centrifuged and incubated at 37°C for 1 hour. This was followed by an incubation at 100°C of 5 minutes before being placed in an ice slurry. 5μl of the same dye as previously used was added to each sample, the samples flick mixed and incubated at 37°C for 1 hour. To stop the reaction, 5μl of 0.5M EDTA was added, the samples incubated at 37°C for 10 minutes and placed in an ice slurry. The experiment was completed in duplicate and the opposing dyes used for reference and tumour sample labelling.

DNA hybridisation to the SpectralChip™ 2600 array

The tumour and reference DNA labelled with the opposite dye were mixed together as illustrated in Figure 2.4. The reference DNA combined with each tumour sample was the opposite sex to that of the tumour patient. In the first arm of the experiment, the tumour DNA was labelled with Cy5 and the reference DNA with Cy3. In the second arm of the experiment, the tumour and reference DNAs were labelled with the opposing dye.

Figure 2.4 Experiment work flow for aCGH DNA preparation.



To each sample mix, 45µl of spectral hybridization buffer I, 12.9µl of NaCl and 130µl of isopropanol were added, the samples flick mixed and incubated at room temperature, in the dark, for 20 minutes. The samples were centrifuged at 5,000rpm for 20 minutes. The supernatant was removed leaving the DNA pellets that appeared purple. A pink or blue pellet suggests DNA labelling was not completely successful or unequal amounts of tumour and reference DNA were used in the initial stages of the experiment. These pellets were not used. Successful pellets were then washed using 500µl of 70% ethanol, flick mixed and centrifuged at max speed for 3 minutes. The supernatant was removed and the pellets allowed to air dry at room temperature, in the dark, for 10 minutes. The pellets were resuspended in 10µl of DEPC-treated water and incubated at room temperature, in the dark, for 10 minutes.

Once completely resuspended, 30µl of spectral hybridization buffer II was added, the samples flick mixed, briefly centrifuged, incubated at 72°C for 10 minutes and then at 37°C for 30 minutes. The SpectralChip™ 2600 array was placed onto a clean work bench with powder free gloves and the sample was pipetted as a single line down the

centre of the glass slide. Using forceps, a 22 X 600mm coverslip was laid over the slide from top to bottom ensuring no air bubbles were visible. The slide was placed into a hybridization chamber, which was then wrapped in foil and sealed a small plastic bag containing a damp paper towel. This was then placed into a humidified chamber and incubated at 37°C for 16 hours.

Post hybridisation washes

The slides were removed from the hybridization chambers and placed in a coplin jar containing 2X SSC-0.5% SDS at room temperature for 10 minutes. The coverslips were removed without peeling or applying force. The slides were then washed in 2X SSC-50% formamide at 50°C for 20 minutes, 2X SSC-0.1% igepal at 50°C for 20 minutes and 0.2 SSC at 50°C for 10 minutes with agitation. After washing, each slide was dipped in 80% ethanol 5 times before being placed in 80% ethanol for 2 minutes. The slides were then blown dry using nitrogen gas and stored in a desiccator in the dark at room temperature until scanning.

Scanning and analysis

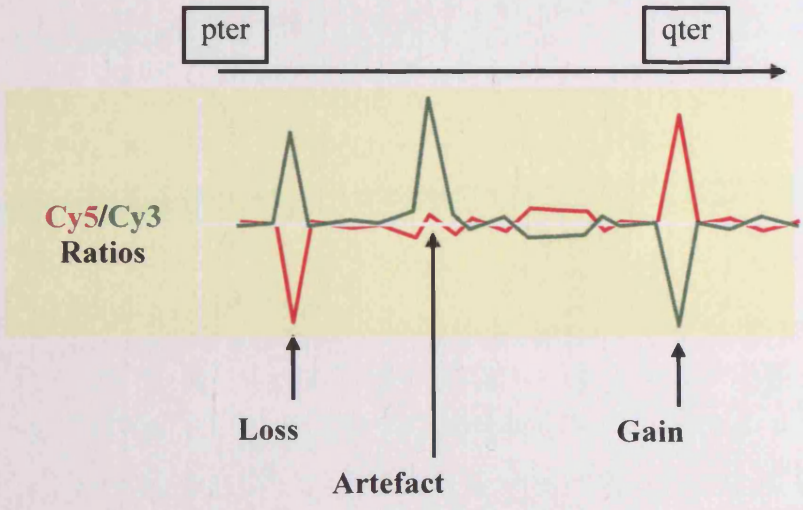
The arrays were scanned using the GenePix Personal 4100A Microarray Scanner (Molecular Devices, CA, USA). Once scanned, images were analysed using GenePix Pro version 6 (Molecular Devices). Image spots were defined by an automatic grid feature that correlated the BAC clone position on the array, with the clone details including genomic locations. The spots were adjusted where necessary and the dye intensities calculated. Further analysis was completed using Formatter Software (Digital Scientific, Cambridge, UK and MIDAS Team (Saeed et al., 2003)). This software programme has previously been used to generate genomic profiles of chronic myelogenous leukaemia and characterize imbalances associated with disease progression (Brazma et al., 2007). Preliminary analysis involved the removal of defective spots and those without a signal. Any spot with an intensity value less than 10000 in one or both channels was discarded. Furthermore, if the background corrected signal intensity was greater than or equal to the background intensity for both channels of a spot, the spot was considered reliable and included in further analysis. A flip-dye consistency check involving the cross-log-ratios of each signal from the duplicated clone spots on each array and from the dye swap experiment was also completed and

those signals that showed a greater than ± 2 SD variation between replicates were discarded (Quackenbush, 2002; Yang et al., 2002).

Iterative linear regression global (whole array) normalisations were completed for each sample reducing the SD of spot $\log_2$ ratios. In this approach, the SD of a given sample was continuously recalculated, removing outliers, until the correlation coefficients converged to a constant value. The final value is referred to as the modified standard deviation (MSD). The outlier range used for this was ± 2 SD (Finkelstein et al., 2002).

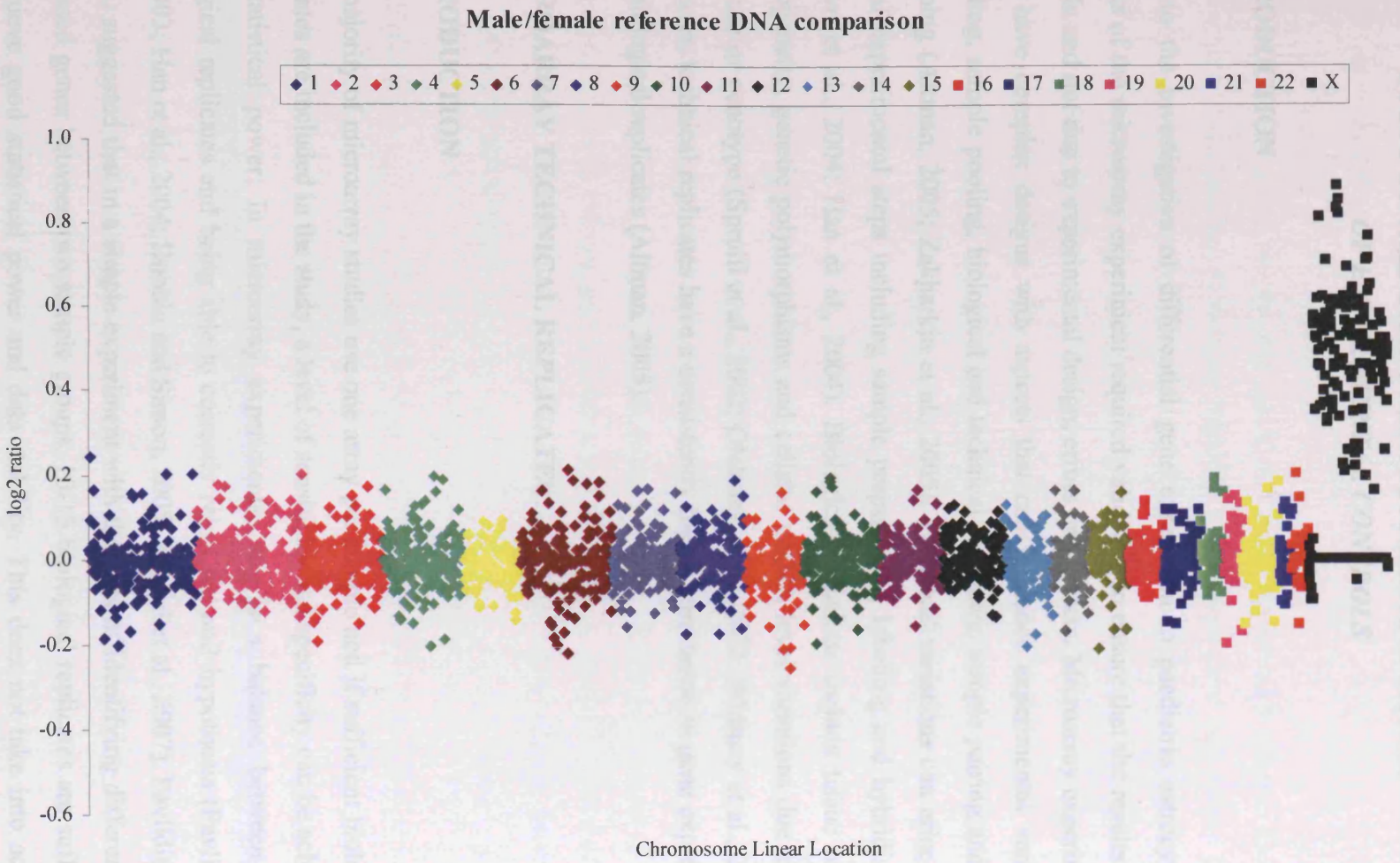
In order to identify those clones that showed gain or loss in the tumour samples, Formatter displays the $\log_2$ ratios that are in excess of a predetermined bar threshold (± 3 MSD was used here). This level assumes that variations in the data are random and constant non-changing results form the majority of data. Theoretically only 0.7% of data values will occur outside the threshold, therefore any deviations exceeding the threshold are considered as true positives (Quackenbush, 2002; Yang et al., 2002). Only results that exceeded the threshold in both arms of the experiment were considered as true, reliable results (Figure 2.5). The effectiveness and reliability of this approach is influenced by the extent of aberrations found in a given sample. Multiple large chromosomal aberrations are excluded during the MSD calculation, therefore fewer constant non-changing results are available for the overall MSD calculations. As a result, the MSD may be unusually large in samples with multiple aberrations and a threshold of 3 SD may not be appropriate.

Figure 2.5 The profile illustrates true chromosome gains and losses compared to experiment artefacts.



The $\log_2$ ratio of the X chromosome was also taken into consideration in each sample analysis. Previous studies have compared the XX and XY $\log_2$ ratio of normal male and female DNA as a marker of copy number alterations. Two studies stated that genomic CNAs in autosomal chromosomes were ± 0.3 $\log_2$ ratios compared to ± 0.68 $\log_2$ ratios for the X chromosome. Consequently threshold values were set at gain > 0.3 $\log_2$ ratio, loss < 0.3 $\log_2$ ratio, amplification > 0.68 $\log_2$ ratio and deletion < 0.3 $\log_2$ ratio (Park et al., 2006; Lee et al., 2007b). An additional study found that the male/female X chromosome $\log_2$ ratio was ± 0.69 (0.05 SD) (Pinkel et al., 1998). The difference in $\log_2$ ratios between autosomes and the X chromosome is thought to be due to cross-hybridisation between some X-chromosome genes and homologous sequences on autosomes (Pollack et al., 1999). A comparison of male and female reference DNA was completed in this study (Figure 2.6). The mean $\log_2$ ratio for the X chromosome was 1.41 (0.15 SD) and no clones showed a greater than ± 0.3 $\log_2$ ratio. A cluster of clones at Xpter show loss in the male/female normal DNA comparison. This alteration is not associated with tumour development and therefore was not considered during analysis. As the X chromosome alterations were taken into consideration when analysing specific tumour profiles, the chromosome was not investigated for aberrations associated with tumour development. However, if the X chromosome $\log_2$ ratio in any tumour experiment showed a significant difference from the control male/female experiment, the chromosome was considered to be altered.

Figure 2.6 This Figure illustrates the aCGH profile comparison of the male and female reference DNA.



CHAPTER 3

THE USE OF BIOPSY AND SHORT-TERM CELL CULTURE SAMPLES IN THE STUDY OF PAEDIATRIC ASTROCYTOMA AND CHOICE OF EXPERIMENTAL CONTROLS

INTRODUCTION

Prior to the investigation of differential gene expression in paediatric astrocytoma, aspects of the microarray experiment required validation to ensure that the results were reliable and not due to experimental design, errors or artefacts. Microarray experiments often have complex designs with aspects that can influence experimental variation including, sample pooling, biological and technical replicates, sample pairing and dye-swapping (Altman, 2005; Zakharkin et al., 2005). Technical variations can arise from several experimental steps including sample preparations, labelling and hybridisation (Brown et al., 2004; Han et al., 2004). Biological variations include tissue or cell heterogeneity, genetic polymorphisms and cellular mRNA level variations due to sex, age, race or genotype (Spruill et al., 2002; Oleksiak et al., 2002; Whitney et al., 2003). Moreover, technical replicates have a consistently higher correlation in gene expression than biological replicates (Altman, 2005).

MICROARRAY TECHNICAL REPLICATES

INTRODUCTION

The majority of microarray studies use one array per sample and if sufficient biological replicates are included in the study, a level of sensitivity and specificity can be achieved for statistical power. In microarray experiments, this is a balance between cost, biological replicates and being able to correctly reject the null hypothesis (Pavlidis et al., 2003; Han et al., 2004; Dobbin and Simon, 2005; Jorstad et al., 2007). Pavlidis et al. (2003) suggested that in a simple experiment with the goal of identifying differentially expressed genes between two sample groups, 10-15 biological replicates are sufficient to achieve good statistical power and data stability. This does not take into account appropriate sample pooling. RNA extracted from specific samples of the same origin (e.g. RNA extracted from individual normal brain tissues) can be pooled together onto a

single array or a smaller number of arrays to reduce experimental costs, overcome the difficulty of limited RNA from individual tissues and reduce gene expression variability between individuals. This approach has been shown to have little effect on statistical power (Peng et al., 2003).

In this study only one microarray chip was to be completed for each sample. This was due to cost and the number of samples included in the study. For this reason it was necessary to confirm that one microarray chip could accurately assess the gene expression profile of a sample and technical replicates were not necessary. Duplicates of 3 short-term cell culture samples were completed using the Affymetrix GeneChip® U133A array. The samples were taken from the same extracted RNA, but the experiments were carried out on different days following the same protocol. This approach was used to assess the impact of technical variation on microarray tumour expression profiles.

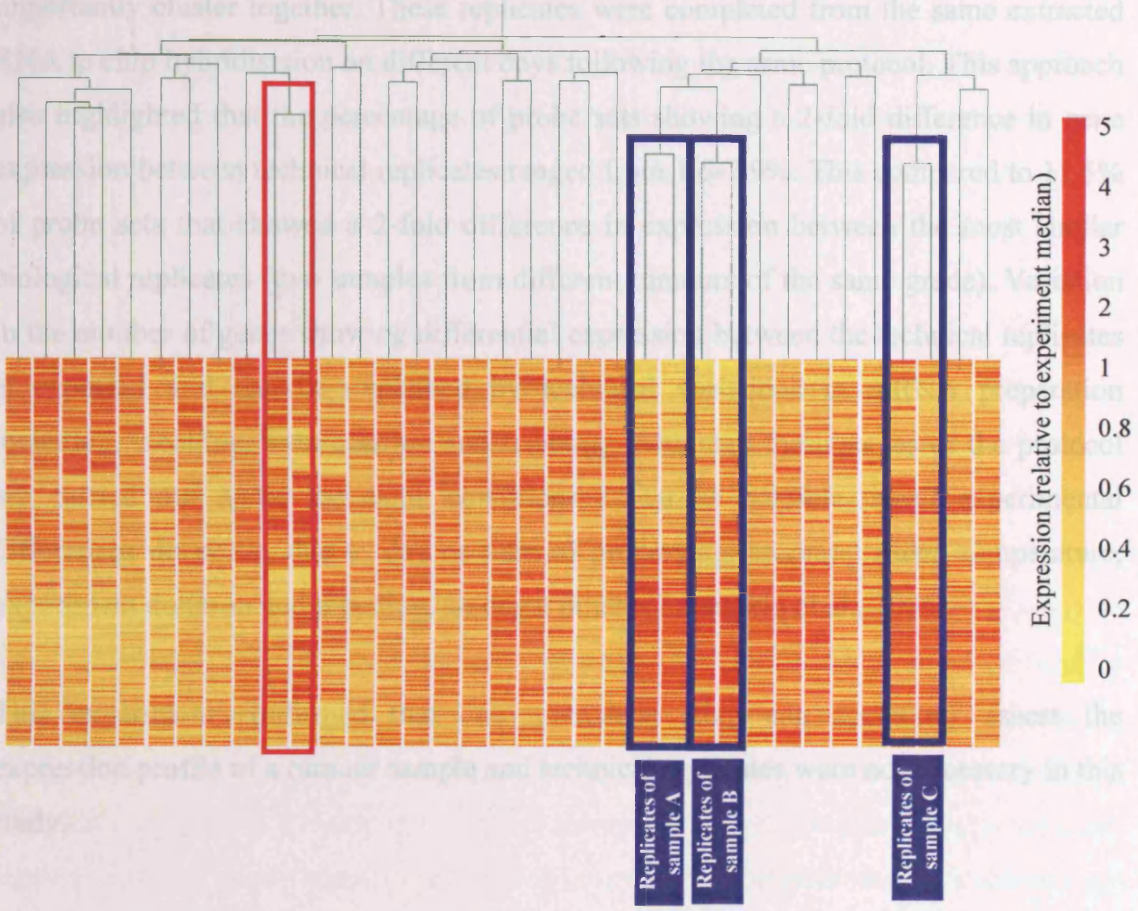
The data from the duplicated samples and an additional 29 samples was grouped in one experiment to assess the reproducibility of the microarray chip. The experiment analysis was carried out as previously described and the mean expression of each gene in all samples was used for the per gene normalisation. Only gene probe sets present in all 35 samples were used for the unsupervised hierarchical clustering approach, a total of 6123 probe sets. This ensured only reliable expression results were used to assess the reproducibility of the arrays.

RESULTS

The replicates of each sample grouped together with a high degree of expression profile similarity (Figure 3.1). The separation ratios were 0.203, 0.178 and 0.177 for the replicates of samples A, B and C respectively (at a separation ratio of 0, gene expression profiles are regarded as identical). This compared to a separation ratio of 0.354 for two tumours that showed the highest expression profile similarity. Furthermore, these tumours are both PAI and therefore can be considered as biological replicates. A similar number of genes showed a 2-fold change in expression between the replicates of samples A and B. However, this was higher than that altered between the replicates of

sample C (Table 3.1). Moreover, 704 probe sets showed a 2-fold difference in expression between the most similar biological replicates, a larger number than that seen between any of the technical replicates.

Figure 3.1 Unsupervised hierarchical clustering of the sample replicates and 29 additional samples.



The replicates of samples A, B and C cluster together with a higher degree of expression profile similarity compared to the remaining samples. The expression profiles of the biological replicates are shown in red.

Table 3.1 The separation ratio and number of genes with a greater than 2-fold change in gene expression between the technical replicates and the biological replicates.

	Separation Ratio	Number of probe sets with a 2-fold difference in expression between replicates	Percentage of genes investigated (%)
Technical replicates of sample A	0.203	417	6.8
Technical replicates of sample B	0.178	481	7.9
Technical replicates of sample C	0.177	99	1.6
Biological replicates of PAI	0.354	704	11.5

DISCUSSION

Unsupervised hierarchical clustering demonstrated that the tumour expression profiles of the technical replicates of 3 samples show a high degree of similarity and most importantly cluster together. These replicates were completed from the same extracted RNA to chip hybridisation on different days following the same protocol. This approach also highlighted that the percentage of probe sets showing a 2-fold difference in gene expression between technical replicates ranged from 1.6-7.9%. This compared to 11.5% of probe sets that showed a 2-fold difference in expression between the most similar biological replicates (two samples from different tumours of the same grade). Variation in the number of genes showing differential expression between the technical replicates is expected and can be explained by technical variations in mRNA preparation including, labelling, hybridisation and scanning. Although these stages of the protocol are carried out under the same conditions as far as possible, small experimental differences do occur, due to day or time of processing, scanning order, temperature, experiment duration and pipetting, amongst others (Zakharkin et al., 2005).

This experiment confirmed that one microarray chip can accurately assess the expression profile of a tumour sample and technical replicates were not necessary in this study.

COMPARISON OF BIOSPY AND SHORT-TERM CELL CULTURE GENE EXPRESSION PROFILES FROM THE SAME TUMOUR

INTRODUCTION

Biopsy tissue, cell lines and short-term cell cultures have been used in the study of tumours and specific studies have directly compared tumour biopsies and short-term cell cultures or cell lines. It is important to recognise that these two tumour cell growth environments, inside the brain or artificial culture conditions, are not equal and this will have some influence on gene expression. It has been shown that the expression profiles of biopsy tissue and cell lines from specific tumours vary greatly, with some cell lines showing profiles that are more similar to the tumour of origin than others. Moreover, several studies have found fewer differences between primary tumour tissue and short-term cell cultures (Ross and Perou, 2001; Virtanen et al., 2002; Sandberg and Ernberg, 2005; Bignotti et al., 2006; Miller et al., 2006; Olsavsky et al., 2007). Two separate studies have compared gene expression profiles from GBMIV tumour tissues and cell lines or derived cultures. Each showed that the cell culture gene expression profiles varied when compared to the tumour tissues and unsupervised hierarchical clustering grouped the cell culture samples independently of the tumour tissues (Hess et al., 2001; Mehrian et al., 2005). Moreover, cellular pathways have been identified in cell lines that show differential gene expression compared to normal and tumour tissues. Pathways up-regulated include ATP synthesis, cell communication pathways, cell cycle, oxidative phosphorylation, purine, pyrimidine and pyruvate metabolism and proteosome pathways and pathways down-regulated include cell adhesion and ECM-receptor interaction (Ertel et al., 2006).

The heterogeneity of tumours and the diversity of short-term cell cultures and cell lines are two elements well recognised in the study of tumours (Nicolson, 1984; Rubin, 1990). Tumours consist of a heterogeneous cell population with various cellular phenotypes existing within an individual tumour and a number of tumours with the same diagnosis. It is this diversity which challenges the study and understanding of tumour growth and development. Furthermore, when tumour cells are cultured, changes are inevitably induced, due to artificial culture conditions. There is also the added

complication of only being able to culture specific tumour cell populations that are able to survive within the artificial environment.

The use of biopsy and short-term cell cultures in the study of paediatric astrocytoma raises several questions:

- Can biopsy and short-term cell culture samples from tumours of the same grade or different grades be compared in the same microarray experiment?
- Can the same sample controls be used for biopsy and short-term cell cultures from the same tumour?

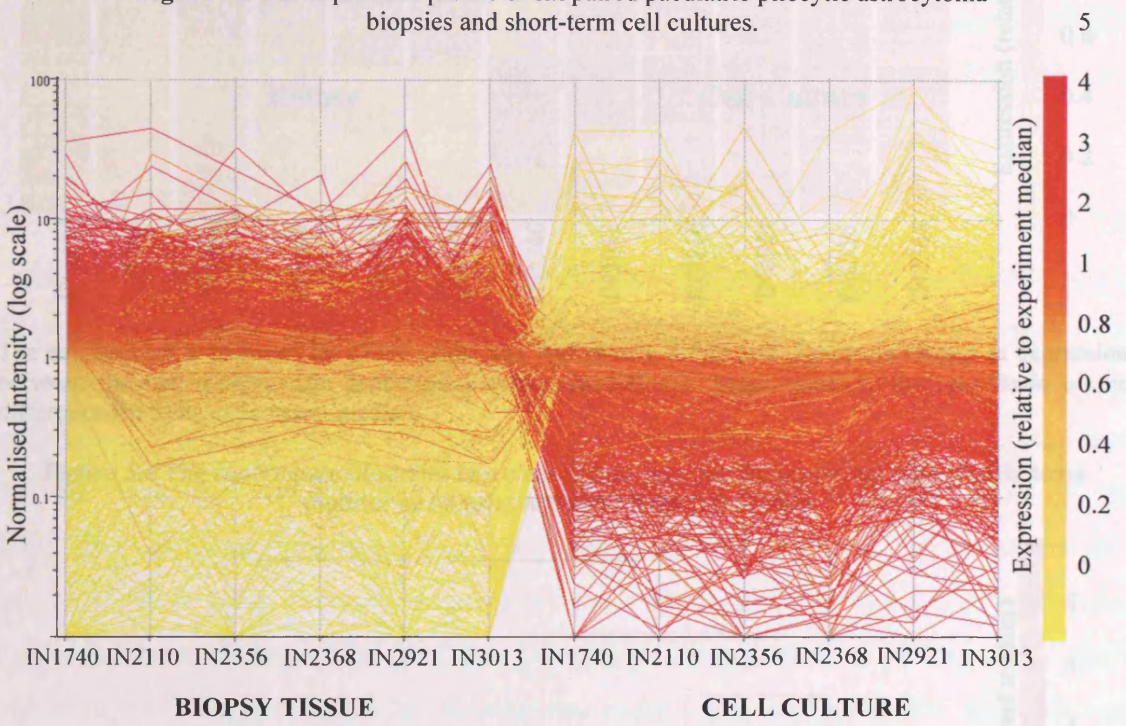
To investigate the use of biopsy and short-term cell cultures in the study of astrocytomas and the most appropriate experiment design when studying these different samples, comparisons were made between the gene expression profiles of 6 paired PAI biopsy and short-term cell culture samples from the same tumour mass and 3 paired adult GBMIV biopsy and short-term-cell culture samples from the same tumour mass.

Two experiments were used in this investigation, the first containing the paediatric PAI samples and the second containing the adult GBMIV samples. The experiment analysis was carried out as previously described and the mean expression of each gene in all samples of each individual experiment was used for the per gene normalisation. Gene probe sets present in 6 of 12 paediatric PAI samples, which corresponded to 11433 probe sets and probe sets present in 3 of the 6 adult GBMIV samples, which corresponded to 12076 probe sets were used for an unsupervised hierarchical clustering approach in each experiment. This allowed those genes that were not expressed in either the biopsy or corresponding short-term cell culture samples to be included in the overall analysis. To compare the biopsy and short-term cell culture samples in this study with those of other studies, further analysis approaches were also completed.

RESULTS

In the experiment containing the 12 paediatric PAI biopsy and short-term cell culture samples, 3157 probe sets showed a 2-fold and significant change in expression between the biopsies and short-term cell cultures (Figures 3.2 and 3.3). An unsupervised hierarchical clustering approach using the 11433 reliable gene expression results clearly demonstrates the clustering of these two sample groups. Moreover, the branching pattern of the dendrogram is different in each group (Figure 3.3).

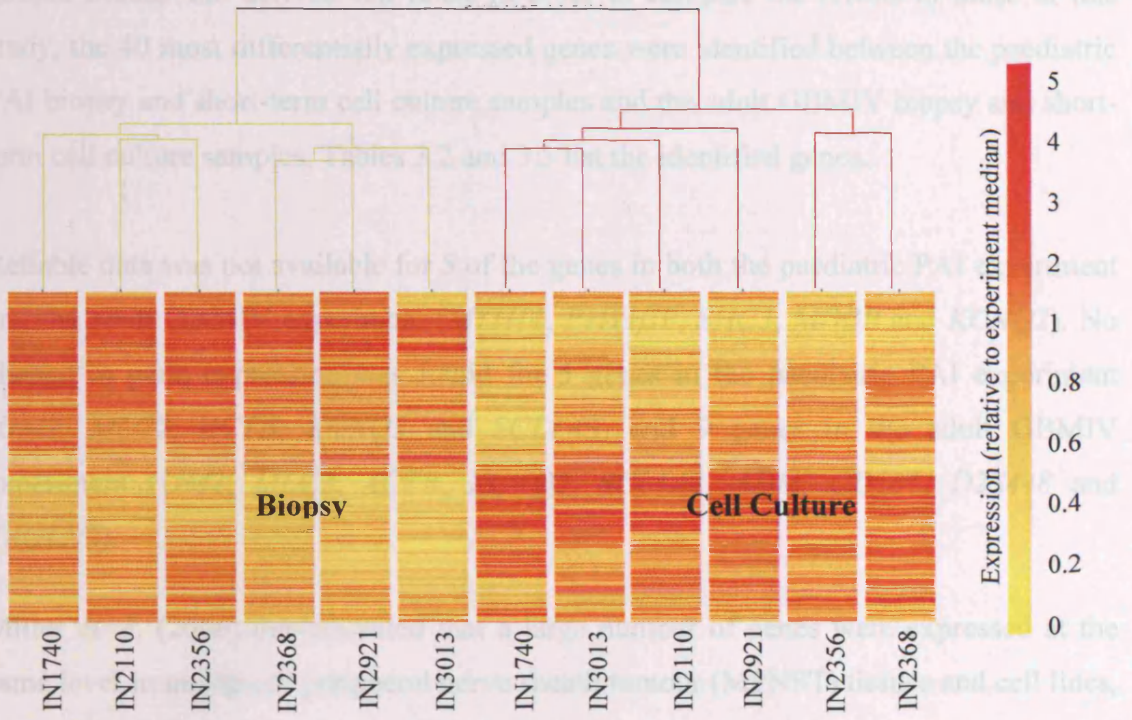
Figure 3.2 The expression profile of six paired paediatric pilocytic astrocytoma biopsies and short-term cell cultures.



The profile illustrates the 3157 probe sets that show a 2-fold and significant change in expression between the paired samples.

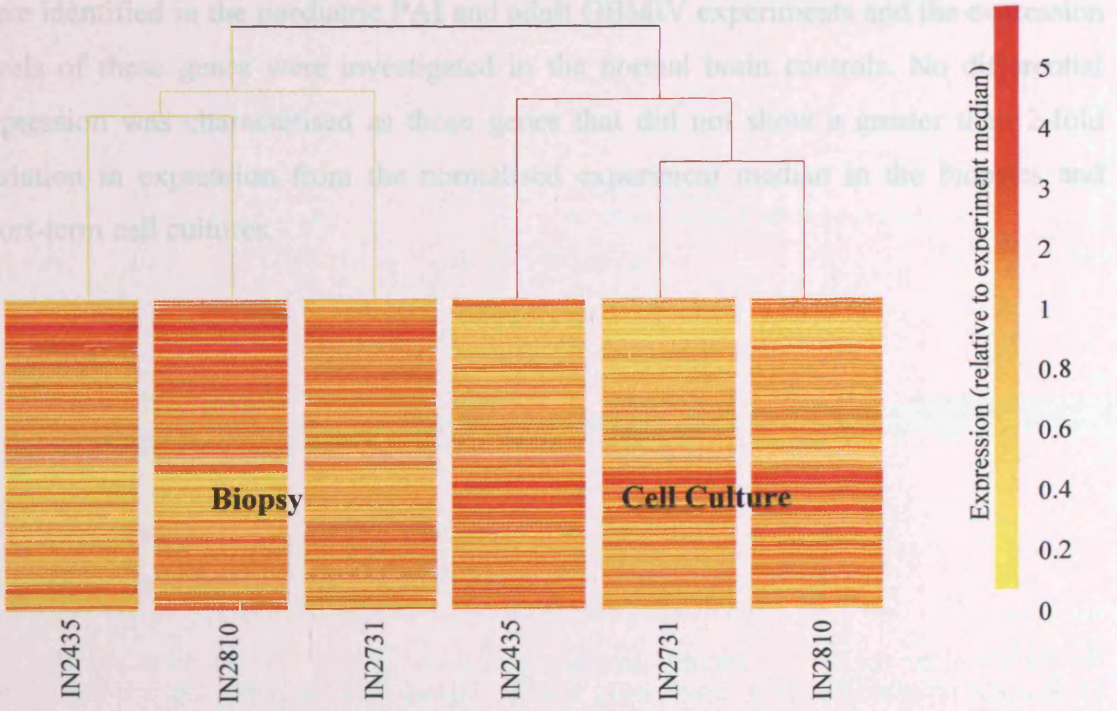
In the experiment containing the 6 adult GBMIV biopsy and short-term cell culture samples, 2629 probe sets showed a 2-fold and significant change in expression between the biopsies and short-term cell cultures (Figures 3.4). The unsupervised hierarchical clustering approach using the 12076 reliable gene expression results, again clearly demonstrates the clustering of these two sample groups. Furthermore, the branching pattern of the dendrogram is different in each group (Figure 3.4).

Figure 3.3 The dendrogram illustrates two clear groups between the six paired paediatric pilocytic astrocytoma biopsies and short-term cell cultures.



The dendrogram is coloured by 11433 probe sets that show a 2-fold and significant change in expression between the two groups. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median.

Figure 3.4 The dendrogram illustrates two clear groups between the six paired adult glioblastoma multiforme biopsies and short-term cell cultures.



The dendrogram is coloured by 12076 probe sets that show a 2-fold and significant change in expression between the two groups. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median.

Mehrian et al. (2005) identified the 40 most differentially expressed genes between glioma tissues and derived cell lines. In order to compare the results to those of this study, the 40 most differentially expressed genes were identified between the paediatric PAI biopsy and short-term cell culture samples and the adult GBMIV biopsy and short-term cell culture samples. Tables 3.2 and 3.3 list the identified genes.

Reliable data was not available for 5 of the genes in both the paediatric PAI experiment and the adult GBMIV experiment (*MYH11*, *PHYHIP*, *SHC3*, *MOBP* and *KCNQ2*). No change in gene expression was found for 5 genes in the paediatric PAI experiment (*UM2*, *MLC2*, *ACTB*, *KCNQ2* and *SCL1A3*) and 9 genes in the adult GBMIV experiment (*UM2*, *MLC2*, *ACTB*, *KCNQ2*, *SCL1A3*, *ADM*, *CD151*, *D2S448* and *TAGLN2*).

Miller et al. (2006) demonstrated that a large number of genes were expressed at the same level in malignant peripheral nerve sheath tumour (MPNST) tissues and cell lines, but were expressed at a different level when compared to controls, which were human Schwann cells (HSC). In order to make the same comparisons between the primary tumours, derived short-term cell cultures and normal controls of this study, genes that showed no differential expression between the biopsies and short-term cell cultures were identified in the paediatric PAI and adult GBMIV experiments and the expression levels of these genes were investigated in the normal brain controls. No differential expression was characterised as those genes that did not show a greater than 2-fold variation in expression from the normalised experiment median in the biopsies and short-term cell cultures.

Table 3.2 Differentially expressed genes between paediatric pilocytic astrocytoma biopsy and short-term cell culture samples.

Ordered position from 3157genes	Fold Change	Gene Name	Description
1	265.8	STC2	stanniocalcin 2
2	207.7	GREM1	cysteine knot superfamily 1, BMP antagonist 1
3	150.2	COL1A1	collagen, type I, alpha 1
4	120.2	FBN1	fibrillin 1 (Marfan syndrome)
5	116.0	MYL9	myosin, light polypeptide 9, regulatory
6	114.6	GPR49	G protein-coupled receptor 49
7	104.3	COL1A2	collagen, type I, alpha 2
8	**94.2**	**CNN1**	**calponin 1, basic, smooth muscle**
9	89.4	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)
10	78.0	COL6A2	collagen, type VI, alpha 2
11	76.6	MYO1D	myosin ID
12	75.7	COL3A1	collagen, type III, alpha 1
13	69.7	FN1	fibronectin 1
14	66.0	LOX	Human lysyl oxidase (LOX) gene, exon 7.
15	65.3	KRT7	keratin 7
16	64.9	CALD1	caldesmon 1
17	**64.2**	**SERPINH1**	**serine (or cysteine) proteinase inhibitor**
18	63.4	THBS1	thrombospondin 1
19	63.1	HSPG2	heparan sulfate proteoglycan 2 (perlecan)
20	60.2	SERPINE1	serine (or cysteine) proteinase inhibitor
23	**55.8**	**PHLDA2**	**tumour suppressing subtransferable candidate 3**
81	**19.7**	**NID2**	**nidogen 2 (osteonidogen)**
83	**19.4**	**ACTG2**	**actin, gamma 2, smooth muscle, enteric**
116	**15.3**	**ADM**	**adrenomedullin**
261	**7.4**	**TRAM2**	**TRAM-like protein**
341	**5.7**	**ANXA2**	**annexin A2**
480	**4.4**	**PFKP**	**phosphofructokinase, platelet**
521	**4.1**	**CD151**	**CD151 antigen**
549	**3.9**	**NBL1**	**neuroblastoma, suppression of tumorigenicity 1**
596	**3.7**	**S100A11**	**S100 calcium binding protein A11 (calgizzarin)**
665	**3.3**	**D2S448**	**Melanoma associated gene**
693	**3.2**	**TAGLN2**	**transgelin 2**
695	**3.2**	**STAT1**	**Homo sapiens transcription factor ISGF-3 mRNA**
711	**3.2**	**MAP2K3**	**mitogen-activated protein kinase kinase 3**
740	**3.1**	**CNN2**	**calponin 2**
1650	**-2.2**	**ARL6IP**	**ADP-ribosylation factor-like 6 interacting protein**
2176	**-3.3**	**SYT11**	**synaptotagmin XI**
2432	**-4.7**	**CNP**	**2',3'-cyclic nucleotide 3' phosphodiesterase**
2459	**-4.8**	**RNASE1**	**Homo sapiens ribonuclease, RNase A family, 1**
2689	**-7.9**	**CCND2**	**cyclin D2**
2824	**-12.7**	**CRMP1**	**collapsin response mediator protein 1**
2915	**-17.5**	**OLFM1**	**olfactomedin 1**
2934	**-19.9**	**RASSF2**	**Ras association (RalGDS/AF-6) domain family 2**
2964	**-24.8**	**SPOCK2**	**sparc/osteonectin,**
3016	**-37.6**	**CLDN5**	**claudin 5 (deleted in velocardiofacial syndrome)**
3065	**-68.5**	**SNAP25**	**synaptosomal-associated protein, 25kDa**
3138	-219.3	PTPRZ1	protein tyrosine phosphatase
3139	-220.3	PLP1	proteolipid protein 1
3140	-222.7	CHL1	cell adhesion molecule with homology to L1CAM
3141	-224.7	GFAP	glial fibrillary acidic protein
3142	-228.3	SNX10	sorting nexin 10
3143	**-228.3**	**GPM6A**	**glycoprotein M6A**
3144	-231.5	ASCL1	achaete-scute complex-like 1 (Drosophila)
3145	-241.0	m6b1	glycoprotein M6B
3146	-243.3	FABP7	fatty acid binding protein 7, brain
3147	**-260.4**	**PMP2**	**peripheral myelin protein 2**
3148	-272.5	NKX2-2	NK2 transcription factor related, locus 2 (Drosophila)
3149	-288.2	PDZK3	PDZ domain containing 3
3150	-306.7	CX3CR1	chemokine (C-X3-C motif) receptor 1
3151	-312.5	S100B	S100 calcium binding protein, beta (neural)
3152	-341.3	GAD1	glutamate decarboxylase 1 (brain, 67kDa)
3153	-366.3	HLA-DRA	major histocompatibility complex, class II, DR alpha
3154	-370.4	GRIA2	glutamate receptor, ionotropic, AMPA 2
3155	-387.6	RGS1	B cell activation gene
3156	-401.6	C3	complement component 3
3157	-574.7	TU3A	TU3A protein

This Table illustrates the 40 most differentially expressed genes between PAI biopsies and short-term cell cultures and lists the genes and fold changes of those genes found to be differentially expressed by Mehrian et al. (shown in bold) (Mehrian et al., 2005). Genes highlighted in red are differentially expressed between both paediatric PAI and adult GBMIV biopsy and short-term cell culture samples.

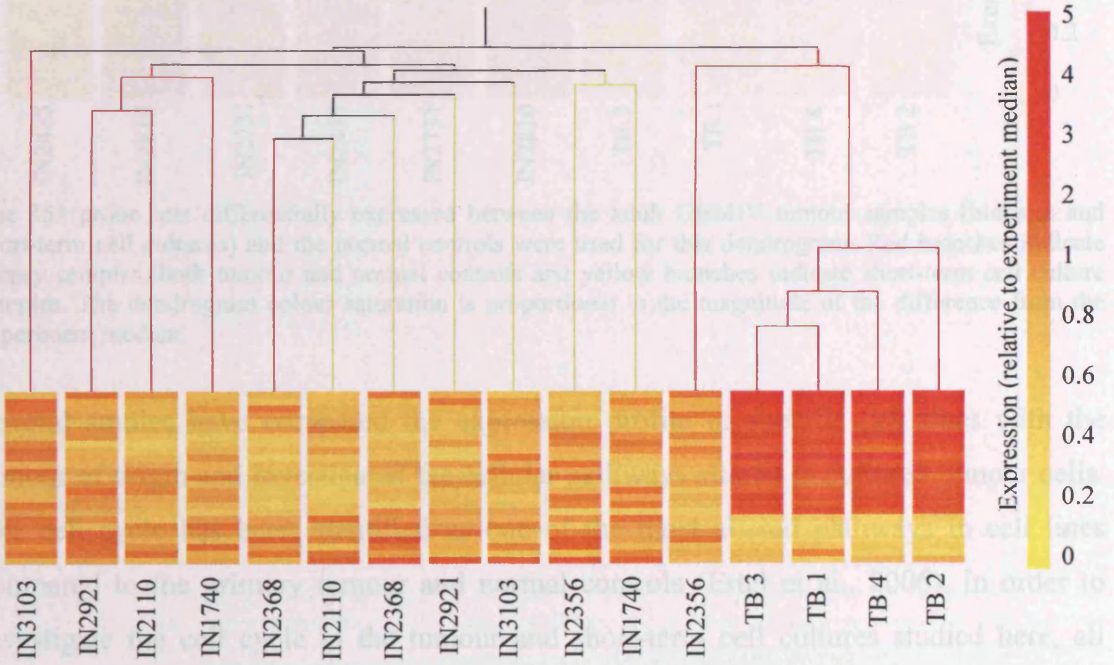
Table 3.3 Differentially expressed genes between adult glioblastoma multiforme biopsy and short-term cell culture samples.

Ordered position from 2969 genes	Fold Change	Gene Name	Description
1	209.1	GREM1	cysteine knot superfamily 1, BMP antagonist 1
2	62.5	PTPLA	protein tyrosine phosphatase-like member a
3	57.5	PTGFR	prostaglandin F receptor (FP)
4	57.2	GATA6	GATA binding protein 6
5	55.7	MET	met proto-oncogene (hepatocyte growth factor receptor)
6	50.4	BST1	bone marrow stromal cell antigen 1
7	43.7	DSP	desmoplakin (DPI, DPII)
8	40.8	STC2	stanniocalcin 2
9	39.0	RGS4	regulator of G-protein signalling 4
10	38.4	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)
11	37.1	WNT5B	wingless-type MMTV integration site family, member 5B
12	36.6	FBN1	fibrillin 1 (Marfan syndrome)
13	34.4	NGFB	nerve growth factor, beta polypeptide
14	34.0	ABLIM3	KIAA0843 protein
15	33.4	GPR49	G protein-coupled receptor 49
16	33.2	PAPPA	pregnancy-associated plasma protein A
17	32.4	THBS1	thrombospondin 1
18	31.9	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
19	30.5	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)
20	30.2	PTGDS	prostaglandin D2 synthase 21kDa (brain)
48	**16.4**	**CNN1**	**calponin 1, basic, smooth muscle**
167	**6.5**	**NID2**	**nidogen 2 (osteonidogen)**
202	**5.6**	**TRAM2**	**TRAM-like protein**
238	**4.9**	**NBL1**	**neuroblastoma, suppression of tumorigenicity 1**
298	**4.2**	**PHLDA2**	**tumor suppressing subtransferable candidate 3**
311	**4.0**	**S100A11**	**S100 calcium binding protein A11 (calgizzarin)**
371	**3.5**	**ACTG2**	**actin, gamma 2, smooth muscle, enteric**
420	**3.2**	**MAP2K3**	**mitogen-activated protein kinase kinase 3**
512	**2.8**	**STAT1**	**signal transducer and activator of transcription 1, 91kDa**
590	**2.6**	**CNN2**	**calponin 2**
645	**2.5**	**ANXA2**	**annexin A2**
670	**2.4**	**SERPINH1**	**serine (or cysteine) proteinase inhibitor**
874	**2.2**	**PFKP**	**phosphofructokinase, platelet**
1362	**-2.4**	**ARL6IP**	**ADP-ribosylation factor-like 6 interacting protein**
1535	**-2.7**	**CNP**	**2',3'-cyclic nucleotide 3' phosphodiesterase**
2166	**-6.6**	**SPOCK2**	**sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2**
2170	**-6.7**	**RNASE1**	**Homo sapiens ribonuclease, RNase A family, mRNA.**
2184	**-6.9**	**CCND2**	**cyclin D2**
2231	**-7.9**	**SYT11**	**synaptotagmin XI**
2234	**-7.9**	**CRMP1**	**collapsin response mediator protein 1**
2235	**-8.0**	**OLFM1**	**olfactomedin 1**
2372	**-12.9**	**SNAP25**	**synaptosomal-associated protein, 25kDa**
2413	**-17.6**	**RASSF2**	**Ras association (RalGDS/AF-6) domain family 2**
2528	**-52.1**	**CLDN5**	**claudin 5**
2610	-143.5	HLA-DPA1	major histocompatibility complex, class II, DP alpha 1
2611	-147.7	BBOX1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1
2612	-152.0	ASCL1	achaete-scute complex-like 1 (Drosophila)
2613	**-155.0**	**PMP2**	**peripheral myelin protein 2**
2614	-159.7	GDF1	Homo sapiens growth differentiation factor 1 (GDF1), mRNA.
2615	-165.3	CD74	CD74 antigen
2616	-165.6	FCGBP	Fc fragment of IgG binding protein
2617	-176.1	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A, member 3
2618	-187.6	CXCR4	Homo sapiens CXCR4 gene encoding receptor CXCR4.
2619	-190.5	PTPRZ1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1
2620	-192.7	GFAP	glial fibrillary acidic protein
2621	**-210.1**	**GPM6A**	**glycoprotein M6A**
2622	-236.4	S100B	S100 calcium binding protein, beta (neural)
2623	-240.4	C3	complement component 3
2624	-243.3	HLA-DRA	major histocompatibility complex, class II, DR alpha
2625	-248.8	m6b1	glycoprotein M6B
2626	-251.9	FABP7	fatty acid binding protein 7, brain
2627	-263.9	TU3A	TU3A protein
2628	-300.3	RGS1	B cell activation gene
2629	-325.7	TTYH1	tweety homolog 1 (Drosophila)

This Table illustrates the 40 most differentially expressed genes between adult GBMIV biopsies and short-term cell cultures and lists the genes and fold changes of those genes found to be differentially expressed by Mehrian et al. (shown in bold) (Mehrian et al., 2005). Genes highlighted in red are differentially expressed between both paediatric PAI and adult GBMIV biopsy and short-term cell culture samples.

In the 12 paediatric PAI tumour samples and 6 adult GBMIV tumour samples, 403 and 2652 probe sets respectively, showed no change in expression. The difference in the number of genes that showed no change in expression between these 2 groups is likely to be influenced by the variation in n number. From these gene lists, those genes that showed a 2-fold and significant change in expression between the tumour sample groups and 4 normal total brain controls were identified. (Refer to Control Samples in this Chapter for the use of this specific normal control). Only 25 probe sets showed differential gene expression between the controls and the paediatric PAI samples and 353 probe sets showed differential gene expression between the controls and the adult GBMIV samples. A hierarchical clustering approach was then used to cluster each tumour group and normal controls, according to the expression profile similarity using these gene probe sets (Figures 3.5 and 3.6).

Figure 3.5 The dendrogram illustrates differential gene expression between paediatric pilocytic astrocytoma tumour samples (biopsies and short-term cell cultures) and normal controls.

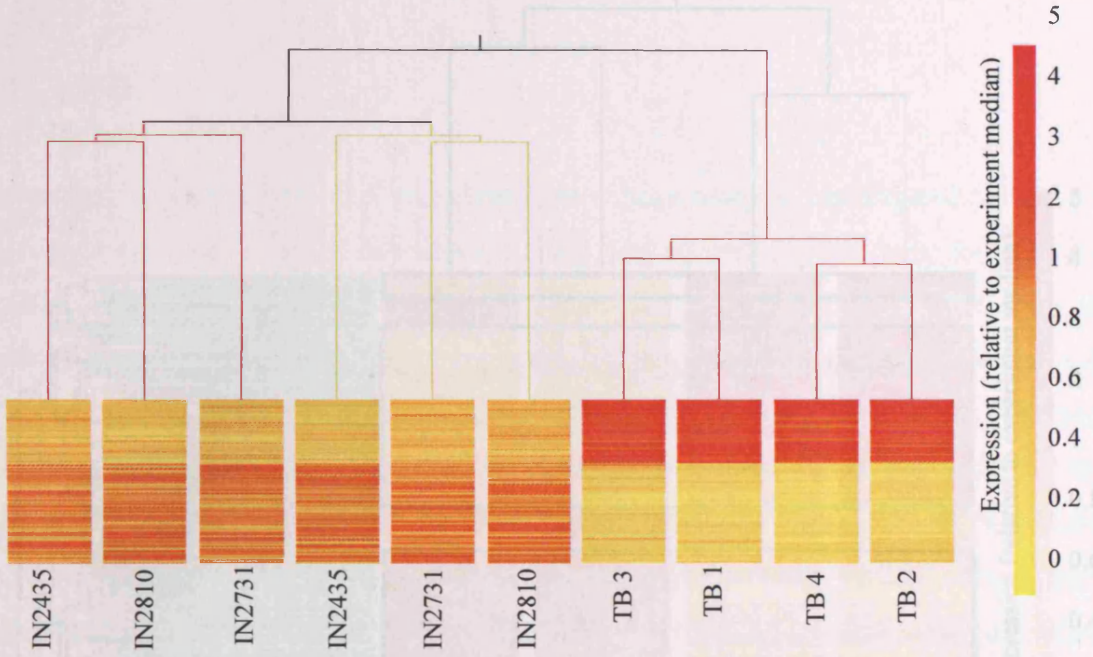


The 25 probe sets differentially expressed between the paediatric PAI tumour samples (biopsies and short-term cell cultures) and the normal controls were used for this dendrogram. Red branches indicate biopsy samples, both tumour and normal controls and yellow branches indicate short-term cell culture samples. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median.

In the dendrogram comparing the 25 probe sets differentially expressed between the normal controls and the paediatric PAI, a distinction can still be seen between the biopsy and short-term cell culture groups although some integration has occurred.

Moreover, 1 biopsy sample clusters with the normal control samples. In the dendrogram comparing the 353 probe sets differentially expressed between the normal controls and the adult GBMIV, a clear distinction can still be seen between the biopsy and short-term cell culture groups.

Figure 3.6 The dendrogram illustrates differential gene expression between adult glioblastoma multiforme tumour samples (biopsies and short-term cell cultures) and normal controls.

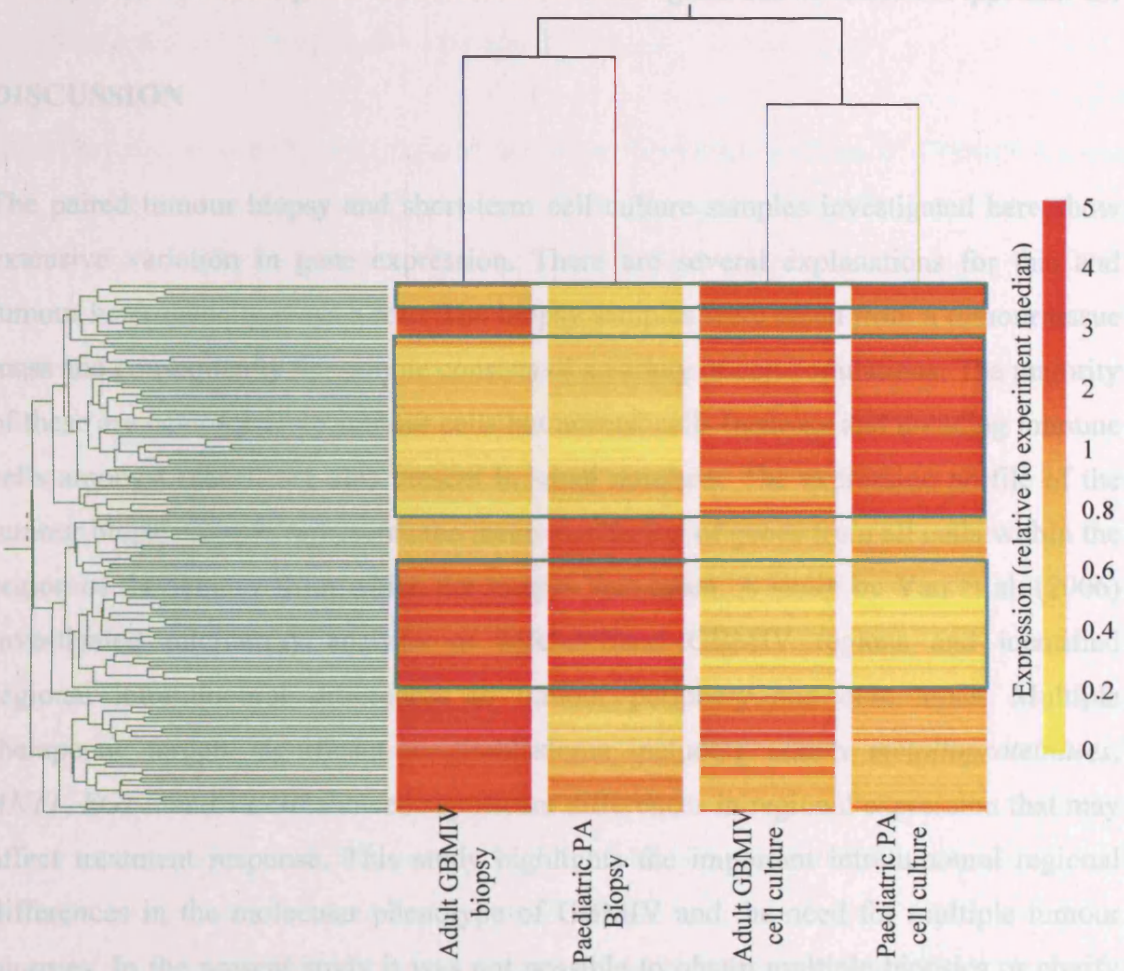


The 353 probe sets differentially expressed between the adult GBMIV tumour samples (biopsies and short-term cell cultures) and the normal controls were used for this dendrogram. Red branches indicate biopsy samples, both tumour and normal controls and yellow branches indicate short-term cell culture samples. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median.

Several studies have compared the expression profile of specific cell lines with the tumour of origin and investigated the cellular pathways altered in cultured tumour cells. The cell cycle has been identified as one of the most altered pathways in cell lines compared to the primary tumour and normal controls (Ertel et al., 2006). In order to investigate the cell cycle in the tumour and short-term cell cultures studied here, all reliable gene expression data for the cell cycle was isolated. In total, 407 cell cycle probe sets were common to both the paediatric PAI and adult GBMIV biopsy and short-term cell culture experiments. A 1-WAY ANOVA statistical significance test ($p = 0.05$) followed by a Benjamini and Hochberg multiple correction test was used to identify those genes that were significantly differentially expressed between the 4 groups comprising; adult GBMIV biopsies, paediatric PAI biopsies, adult GBMIV short-term cell cultures and paediatric PAI short-term cell cultures. In total, 126 probe sets were

significantly differentially expressed between the 4 sample groups. The sample groups and gene expression similarities are illustrated in the dendrogram of Figure 3.7.

Figure 3.7 The dendrogram illustrates 126 differentially expressed cell cycle genes between paediatric pilocytic astrocytoma and adult glioblastoma multiforme biopsies and short-term cell cultures.



The 126 probe sets that were differentially expressed between the paediatric PAI and adult GBMIV biopsies and short-term cell cultures are clustered (branches to the left of the dendrogram) according to gene expression similarity. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median. Several clear cell cycle gene groups can be observed and are indicated by the green lines.

Moreover, although the biopsy and short-term cell culture samples cluster together and it is clear that a large number of genes are differentially expressed between these sample types, all 4 profiles show similarities and differences (indicated by the positioning of the green lines in Figure 3.7). The genes that cluster at the bottom of the dendrogram show higher expression in the adult GBMIV biopsies compared to the remaining 3 groups, with some of these genes showing a slightly higher expression in the adult GBMIV

short-term cell cultures. A small cluster of genes in the middle of the dendrogram show a higher expression in the PAI biopsies compared to the remaining 3 groups and a cluster of genes towards the top of the dendrogram show reduced expression in the paediatric PAI biopsies compared to the remaining 3 groups. Together these 126 genes show a molecular signature that can distinguish biopsy and cell culture samples. The details of the cell cycle genes involved in this dendrogram can be found in Appendix III.

DISCUSSION

The paired tumour biopsy and short-term cell culture samples investigated here show extensive variation in gene expression. There are several explanations for this and tumour heterogeneity plays a role. The biopsy samples were taken from a tumour tissue mass and consequently the sample consists of a variety of cell populations. The majority of these are heterogeneous tumour cells but normal cells (neurons and invading immune cells amongst others) are also present in small numbers. The expression profile of the tumour biopsy sample represents the mean expression of genes from all cells within the region of the tumour from which the sample was taken. A study by Van et al. (2006) investigated microarray analysis of MRI-defined GBMIV regions and identified regional intratumoural differences in tumour periphery and core areas. Multiple therapeutic targets significant to glioblastoma including *matrix metalloproteinases*, *AKT1*, *EGFR* and *VEGF* showed significant differences in regional expression that may affect treatment response. This study highlights the important intratumoural regional differences in the molecular phenotype of GBMIV and the need for multiple tumour biopsies. In the present study it was not possible to obtain multiple biopsies or clarify the tumour region from which the biopsy samples were taken. Consequently, this is a source of variation within the biopsy samples investigated here.

The short-term cell culture samples studied here were grown from the tumour biopsy tissue, as previously described in Chapter 1. The heterogeneity of tumour tissue and the artificial growth environment does select for cell sub-populations that are able to grow under artificial culture conditions. The gene expression profile of the short-term cell culture samples investigated in this study represents a cell population of the paediatric PAI or adult GBMIV tumours. However, the profile may not represent the majority cell population of the heterogeneous tumour tissue. This introduces variation between the

cell culture samples, which is reflected in the biopsy and short-term cell culture clusters illustrated in the dendrograms of Figures 3.3 and 3.4. The clusters do not show the same branching patterns and different samples group together, highlighting the variation between the samples of each group. Furthermore, it is also well known that long-term cell culture induces further expression and genomic changes in the cultured cell population (Band et al., 1990; Sen et al., 1995). For this reason, all cultures in this study are short-term cell cultures below passage 8.

Two previous studies have compared the gene expression profiles of GBMIV tissues and cell lines. The study by Hess et al. (2001) compares GBMIV tissue expression profiles with well established GBMIV cell lines. The study by Mehrian et al. (2005) investigates the expression profiles of glioma tissues and derived cell lines of approximately passage 10. The results found by these two studies are similar to those found here. In each study, the tumour tissue and cell culture samples group independently when clustered according to expression profile similarity. Of the 40 genes identified by Mehrian et al. that showed the most differential gene expression between the glioma tissues and derived cell lines, thirty showed a similar expression pattern between the paediatric PAI and adult GBMIV biopsy and short-term cell cultures studied here (Tables 3.2 and 3.3).The genes found to be up-regulated in this group are associated with nucleotide and nucleic acid metabolism, cell proliferation and signal transduction. Those down-regulated include genes involved in development, signal transduction, and transporter activity. Mehrain et al. (2005) suggested that GBMIV tumour cells selected during culture have a more rapid proliferation rate and a more undifferentiated phenotype compared to the primary tumour cells. In both the paediatric PAI biopsy and short-term cell culture comparisons and the adult GBMIV biopsy and short-term cell culture comparisons of this study, the 40 most differentially expressed genes were involved in nervous system development, cell differentiation and G-protein coupled receptor signalling in. Furthermore, genes involved in immune response were only differentially expressed between the adult GBMIV biopsies and short-term cell cultures and genes involved in cell adhesion and phosphate transport were only differentially expressed in the paediatric PAI biopsies and short-term cell cultures.

Moreover, the majority of the 40 genes that were most differentially expressed genes between the paediatric PAI and adult GBMIV biopsy and short-term cell cultures of this study were different to those found by Mehrian et al. (2005). This may be because the samples investigated here are short-term cell cultures of approximately passage 8. Moreover, although not stated in the analysis protocol used by Mehrian et al., those genes present in all tissue samples and absent in all short-term cell culture samples or vice versa, do not appear to be included in the analysis. Consequently, the experiment approach of this study used a larger number of genes for further analysis. This would explain the differences found by each study as well as the correlations. It was also noted that GFAP expression was significantly down-regulated in the short-term cell culture samples compared to the tumours tissue. This has previously been documented in short-term cell cultures by approximately passage 5/6, derived from 50 astrocytomas (Lolait et al., 1983).

A further study compared the expression profiles of HSC, MPNST and cell lines. A molecular signature of 209 probe sets was characterised that distinguished MPNST primary tumours and cell lines from normal HSC (Miller et al., 2006). This is not a complete profile of global gene expression as two different Affymetrix GeneChips® were used in this study and only those genes common to both arrays could be compared. Of the 209 probe sets, 159 genes clustered into 3 groups that validated the use of MPNST cell lines as an *in vitro* model of MPNST primary tumours. The first cluster contained 10 genes that were up-regulated in the MPNST cell lines compared to the primary tumours. The protein products of the majority of these genes are involved in cell cycle progression. The authors suggest that this is not unexpected and can be attributed to the fact that the cell lines are exposed to non-physiological growth-promoting conditions *in vitro*. Cluster two contained 13 genes that were up-regulated in both the cell lines and primary tumours compared to the HSC and have been implicated in other tumour systems. The remaining 146 genes clustered in the third group and showed a common down-regulation in the MPNST samples compared to the HSC. A number of these genes are reported to be Schwann cell differentiation markers suggesting that the MPNST cells do not show characteristics of differentiated Scwann cells. (Miller et al., 2006). Furthermore, although the 159 genes showed differential gene expression between the tumour samples as a group and the HSC, hierarchical

clustering distinguished the cell lines and primary tumours and no tumour integration was observed.

Using a similar approach to the study carried out by Miller et al. expression signatures of 25 and 353 probe sets were identified in paediatric PAI and adult GBMIV respectively that showed a significant difference in expression between the tumour samples, regardless of biopsy or short-term cell culture origin and normal brain. In total, 11 genes were common to both experiments. In the paediatric PAI, 7 genes were down-regulated compared to the normal controls and 18 were up-regulated. In the adult GBMIV, 136 probe sets were up-regulated compared to the normal controls and 217 down-regulated. KEGG pathway analysis of the 353 probe sets revealed that genes involved in phosphatidylinositol signalling, MAPK signalling, apoptosis, tight junctions, notch signalling and hedgehog signalling were down-regulated in the adult GBMIV compared to normal controls. Furthermore, genes also involved in phosphatidylinositol signalling and MAPK signalling showed up-regulation as well as genes involved in calcium signalling, gap junctions and VEGF signalling. This approach was not completed for the 25 differentially expressed genes in paediatric PAI compared to normal controls as the gene list was not large enough.

Although the adult GBMIV biopsy and short-term cell culture samples clustered together, sub-branches within this cluster group clearly distinguished the two sample types, as seen in the MPNST tissue, cell line and HSC comparison. Moreover, this was not seen in the hierarchical clustering approach involving the paediatric PAI tumour samples and normal controls, in which tumour biopsies and short-term cell culture samples showed some integration. This biopsy and short-term cell culture integration is most likely due to the small number of genes involved in the genetic signature used for this approach. However, it should be acknowledged that biopsy sample contamination with a small amount of normal tissue may influence gene expression results. Consequently when small numbers of genes are used for hierarchical clustering, specific tumour samples may cluster with normal controls as seen in Figure 3.5.

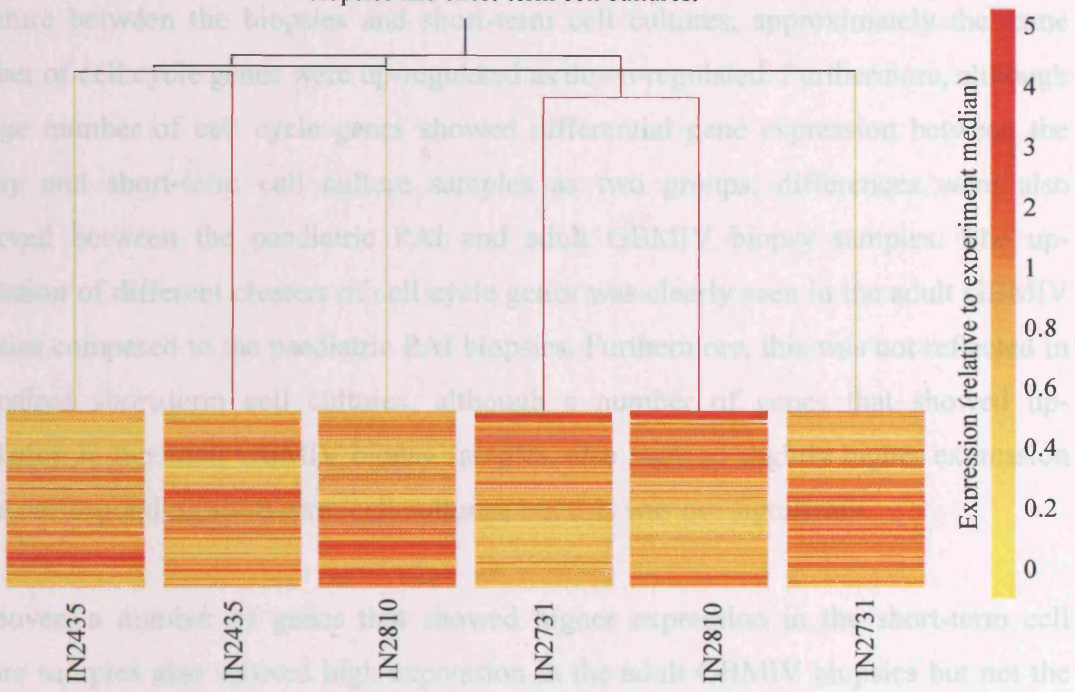
Other studies have compared the expression profiles of specific tumour tissues and either cell lines or derived short-term cell cultures to investigate the impact of cell culture on gene expression. A study investigating 44 different lung tumours and 38

corresponding cell lines found that hierarchical clustering grouped the lung tumour tissue samples and cell lines independently. Within the tissue branch of the dendrogram, all small cell lung carcinoma (SCLC) tissues clustered on one branch and the non-small cell lung carcinoma tissues (NSCLC) clustered on a different branch. The NSCLC branch of the dendrogram showed sub-branches, separating adenocarcinomas (AC), squamous cell carcinomas (SCC) and large cell carcinomas. In the cell line branch of the dendrogram, the cell lines generally clustered according to tumour type and separation of SCLC and NSCLC cell lines was observed. However, within the NSCLC branch of the dendrogram only the SCC cell lines showed a distinct cluster. In order to promote integration of tumour tissues and cell lines when clustered, a filtering algorithm was used to remove genes from the analysis that showed a significant differential expression between the two groups. Moreover, when hierarchical clustering was repeated, the lung tumour and cell line samples did integrate to some extent. Of the 38 cancer cell lines, 24 integrated with the tissue samples and the remaining 14 formed an independent cell line cluster. Within the dendrogram, no particular pathological type clustered independently. However, overall the SCLC and NSCLC tumour and cell line samples did cluster separately (Virtanen et al., 2002).

When this approach was applied to the tumours of the present study and genes significantly differentially expressed between the astrocytoma biopsy and short-term cell culture samples were removed from the hierarchical clustering approach, the paediatric PAI biopsy and short-term cell culture samples did not integrate (data not shown). However, some integration was observed between the biopsy and short-term cell cultures of the adult GBMIV samples (Figure 3.8).

It is possible that the cells derived from the primary adult GBMIV tissues are the majority cell in the tumour and are consequently more representative of the tumour tissue, than those cultured from the PAI biopsies. Furthermore, the tumour induced changes within these cells may result in cells that are more amenable to *in vitro* conditions and less expression changes are consequently induced. Moreover, in the experience of this laboratory, the culture of adult GBMIV cells is more successful than paediatric PAI cells and the doubling times for these tumour cells are higher. These explanations describe possible factors that may influence the integration of adult GBMIV samples compared to paediatric PAI samples.

Figure 3.8 The dendrogram illustrates integration of the adult glioblastoma multiforme biopsies and short-term cell cultures.



The 9447 probe sets that were not differentially expressed between the biopsy and short-term-cell cultures were used for this hierarchical clustering approach and colour the dendrogram. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median. Yellow dendrogram branches indicate short-term cell culture samples and red branches indicate biopsy samples.

Several studies have identified cellular pathways that are differentially expressed between cell lines and primary tumours. A study by Sandberg et al. (2005) compared 60 tumour cell lines from 9 tissue origins with 135 human tissues and 176 tumour samples. The study found that approximately 30% of investigated genes showed a significant difference in expression between the cell lines, tumour and normal tissues, the majority of which were involved in cell cycle progression, macromolecule processing, ATP synthesis, cell adhesion and membrane signalling. Furthermore, a smaller study also identified ATP synthesis, cell communication pathways, cell cycle, oxidative phosphorylation, purine, pyrimidine and pyruvate metabolism and proteosome pathways as pathways up-regulated and cell adhesion molecules and ECM-receptor interaction as pathways down-regulated (Ertel et al., 2006).

This study compared the impact of short-term cell culture on cell cycle gene expression in adult GBMIV and paediatric PAI with the results from the two previous studies as it was the most altered pathway. Ertel et al. (2006) showed that in a common molecular signature of 6 cell lines compared to 6 tumour tissues, the majority of cell cycle genes

were up-regulated. The results the present study found that in a common molecular signature between the biopsies and short-term cell cultures, approximately the same number of cell cycle genes were up-regulated as down-regulated. Furthermore, although a large number of cell cycle genes showed differential gene expression between the biopsy and short-term cell culture samples as two groups, differences were also observed between the paediatric PAI and adult GBMIV biopsy samples. The up-regulation of different clusters of cell cycle genes was clearly seen in the adult GBMIV biopsies compared to the paediatric PAI biopsies. Furthermore, this was not reflected in the paired short-term cell cultures, although a number of genes that showed up-regulation in the adult GBMIV biopsy samples, also showed slightly higher expression in the corresponding short-term cell cultures, but this was not significant.

Moreover, a number of genes that showed higher expression in the short-term cell culture samples also showed high expression in the adult GBMIV biopsies but not the paediatric PAI biopsies. Although paediatric PAI and adult GBMIV cells grow equally well in culture once established, these results highlight the fact that *in vitro* culture changes the phenotype of paediatric PAI cells to a greater extent compared to adult GBMIV cells, by promoting their growth above that of *in vivo* cells which are relatively slow growing. Adult GBMIV cells are already proliferating at a high rate *in vivo,* therefore the growth promoting effects of *in vitro* culture are less obvious.

A study by Wang et al. (2006a) compared 59 cell lines with the presumed tumour of origin. This study defined a cell line as being representative of its tumour of origin if there was no other tumour type that had a gene expression profile with a significantly higher correlation to the cell line than the presumed tumour of origin ($p < 0.05$). Using this approach 51 of the 59 cell lines were representative of the tumour of origin. In the study carried out here, only the expression profiles of short-term cell cultures and astrocytoma biopsy tissues have been compared. If other tumour types had also been included in this study, along with the astrocytoma, a distinction may have been seen between the short-term cell cultures and primary tumours of astrocytoma compared to other tumour types, as seen in the previous study.

It is clear that biopsies and short-term cell cultures from the same tumour mass show different gene expression patterns. There is also no doubt that cells in culture undergo

associated changes in gene expression and this should be recognised when studying tumour short-term cell cultures. The variation in clustering patterns of the specific biopsy and short-term cell culture groups is likely to be a reflection of different tumour cell clones grown from the tumour tissue and the variation in the tumour region used when obtaining the tumour biopsy sample. Moreover, this evidence suggests that it is inappropriate to include a mixture of biopsy and cell culture samples as a representation of astrocytoma in the same experiment, but that the each sample type may be used for the study of paediatric astrocytoma, taking into account the merits and shortcomings in each case.

CONTROL SAMPLES

INTRODUCTION

The choice of normal control samples for microarray experiments investigating gene expression in brain tumours is difficult and sometimes limited. Normal tissue from adjacent normal brain regions of a tumour is not available from most patients, as tumour tissue is usually obtained for diagnosis during resection and it would be inappropriate to sample normal brain at this time. The options available are then reduced to normal brain from other individuals. This introduces variation between tumour samples and normal controls due to variations in the human population and tumour locations (Oleksiak et al., 2002; Morley et al., 2004).

An individual study has investigated the choice of normal ovarian controls and the influence this may have on the determination of differentially expressed genes in ovarian cancer expression profiling studies (Zorn et al., 2003). The study compared five normal control sources (two or three replicates) including, whole ovary samples (WO), ovarian surface epithelium (OSE) and immortalised OSE cell lines with a group of 24 serous ovarian carcinoma samples. The gene lists produced by the comparison of each normal group with the tumour samples were unique to that normal-carcinoma comparison. No gene appeared in all five lists. The study concluded that the OSE brushings were the most appropriate control sample for serous ovarian carcinoma analysis, as they were not cultured and provided the purest samples of OSE (Zorn et al., 2003). The study also recognised that although OSE brushings were felt to be the most appropriate control there was not a perfect control.

Aside from these findings, the study makes two important statements; (i) controversy surrounds the decision to collect normal tissue from the same patient as the tumour or from a different patient thought to be disease free. The adjacent normal approach limits the inclusion of genes that are differentially expressed due to interpatient variability but have little impact on malignant transformation, where as the normal donor approach recognises that even normal-appearing tissue in a diseased individual may exhibit gene expression changes from truly normal tissue; (ii) studies whose aim is to establish overall molecular profiles, such as comparison of tumour profiles with clinical

outcomes, would be less affected by these concerns because referring to a normal control is unnecessary (Zorn et al., 2003).

It is also important to recognise that normal tissue has a different cellular content, morphology and function compared to tumour tissue in the same environment. Although tumour tissue is heterogeneous, normal tissue is a balanced mixture of cell types, each contributing to normal tissue function. This again highlights the question: Is there a good control for the study of tumours?

The normal controls used in previous microarray studies of brain tumours vary greatly. Studies investigating differential gene expression between tumour grades or primary and recurrent tumours have not necessarily included any normal brain samples in the experiments (Kim et al., 2002b; Nutt et al., 2003; van den et al., 2003; Mischel et al., 2003; Sharma et al., 2006). Other studies comparing tumour expression profiles to normal brain have used extracted total RNA from tissue samples obtained from normal brain regions including tissue from a lobectomy after edema, hippocampus tissue from an epileptic patient, subcortical white matter, cortical brain, the cortex of temporal lobe and post-mortem cortex and medulla tissues (Huang et al., 2000; Rickman et al., 2001; Khatua et al., 2003; Shai et al., 2003; Godard et al., 2003). Groups who have not had direct access to normal brain have also commercially purchased total RNA samples of adult and foetal normal brain for use as controls (Godard et al., 2003; Wong et al., 2005).

The use of a variety of normal controls in microarray experiments raises several questions:

- Is it appropriate to compare the expression profile of a tumour located in a specific brain region to a normal brain control taken from another region?
- Should every tumour be individually compared to a normal control from the same location?
- Which normal control is most appropriate for a tumour spanning several brain regions?

- Is age important when comparing controls and tumours from different age groups?

From the previous investigation it was clear that paediatric astrocytoma biopsies and short-term cell cultures cannot be combined in the same experiments in order to study this tumour. Consequently, different controls are necessary for each experiment. Several controls were investigated to ensure that the most appropriate control was selected for each experiment.

It was not possible to obtain normal brain samples from adjacent normal brain regions to the tumour. As an alternative, normal human adult and foetal brain total RNA from total and specific brain regions were purchased as pooled samples from several commercial companies, AMS Biotechnology, United States Biologicals and BD Biosciences. Normal samples were not available from individuals of a closer age to the patient group, therefore both foetal and adult normal brain were investigated as possible controls. These included adult and foetal total brain, corpus callosum and cerebellum.

Foetal astrocytes were obtained as normal controls for the short-term cell culture samples of this study from Cambrex Bio Sciences and Professor Geoff Pilkington at Portsmouth University. No adult astrocytes were available. All controls were compared to six paired paediatric PAI biopsy and short-term cell culture samples and three paired adult GBMIV biopsy and short-term cell culture samples. These tumours were used to investigate the controls as paired tumour biopsy and short-term cell culture samples were available.

Analysis was carried out as previously described and the mean expression of each gene in all samples of the experiment was used for the per gene normalisation. The same 6 paediatric PAI paired biopsy and cell culture samples and 3 adult GBMIV paired biopsy and cell culture samples used in the previous analysis comparing biopsy and short-term cell cultures were included in this analysis. Gene probe sets present in all samples were used for an unsupervised hierarchical clustering approach, a total of 5304 probe sets.

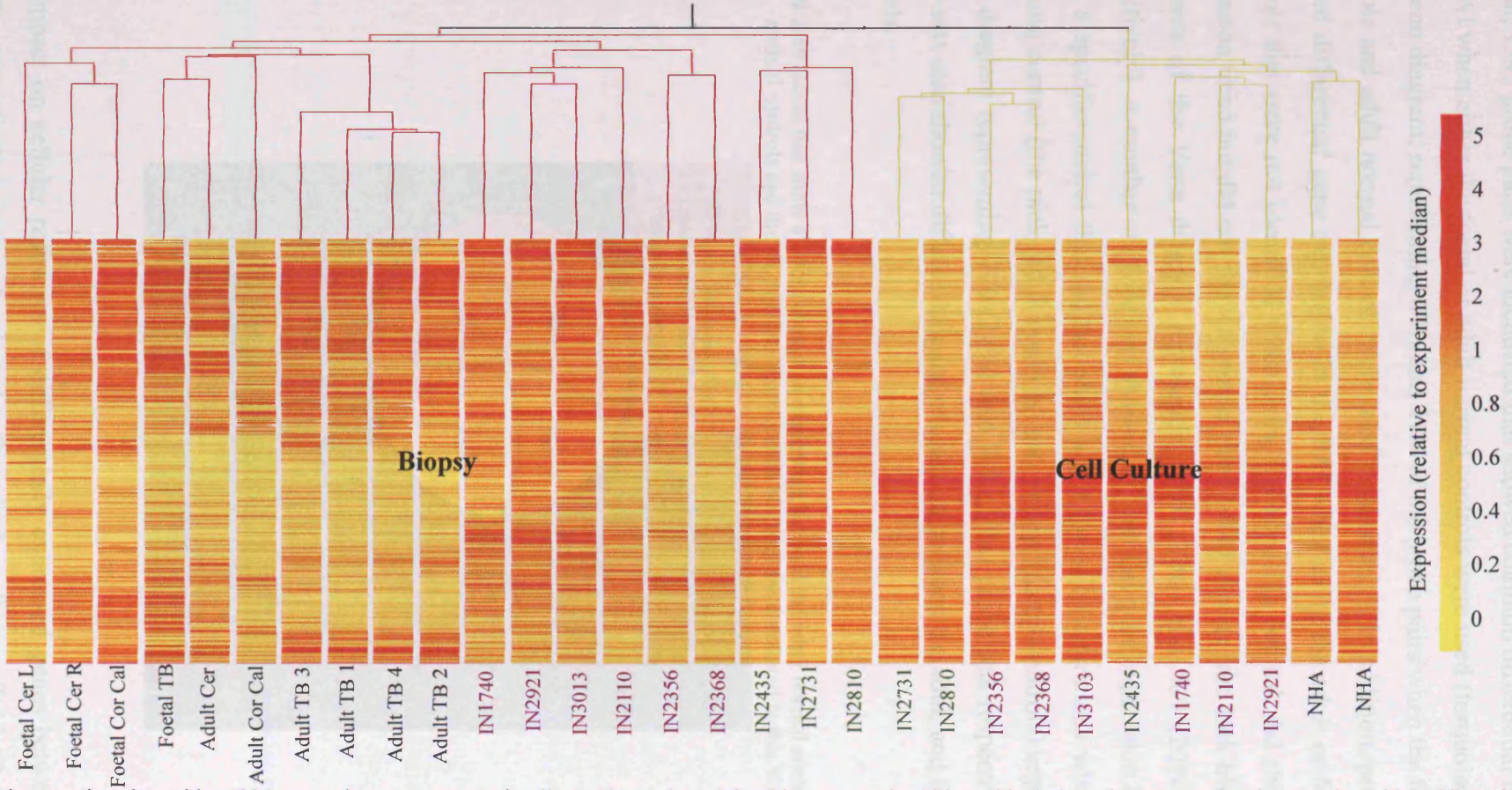
RESULTS

Figure 3.9 illustrates the unsupervised hierarchical clustering approach and gene expression profile similarity between all controls, biopsy and short-term cell culture samples and clearly demonstrates independent sample grouping. Within the biopsy group, the normal brain controls and 6 paediatric PAI biopsies branch independently of the adult GBMIV biopsies. However, the clustering of the 6 paediatric PAI biopsies is separate from the normal brain control samples as a group. Furthermore, within the normal brain control cluster, sub-groups of adult and foetal controls exist, demonstrating that the adult and foetal normal tissue parameter influences the clustering of these samples to a greater extent than the brain region of origin. Variation can also been seen between the expression profiles of tissues from different brain regions.

Within the cell culture group, clustering of the short-term cell cultures is not independent of the normal controls and the paediatric PAI and adult GBMIV short-term cell cultures do not cluster into distinct groups. Moreover, the expression profiles of the short-term cell culture samples as a group (normal astrocytes, paediatric PAI and adult GBMIV short-term cell cultures) are more similar, compared to the corresponding samples in the biopsy group.

The gene expression profiles of the adult and foetal normal brain samples were compared to determine the most suitable control for the tumour biopsies of this study. Those genes that showed a greater than 2-fold and significant change in expression between the six paediatric PAI biopsies and the adult normal controls, the six paediatric PAI biopsies and the foetal normal controls and the foetal and adult normal controls were identified; 3088, 3240 and 2527 probe sets respectively (Figure 3.10). The adult GBMIV biopsies were not combined in this approach in order to reduce variation within the tumour biopsy group. However, a separate approach was completed for the adult samples comparing the three adult GBM GIV biopsies and the adult normal controls and the three adult GBM GIV biopsies and the foetal controls. The data from this approach is not shown as the findings and conclusions made were comparable to those found using the PAI biopsies.

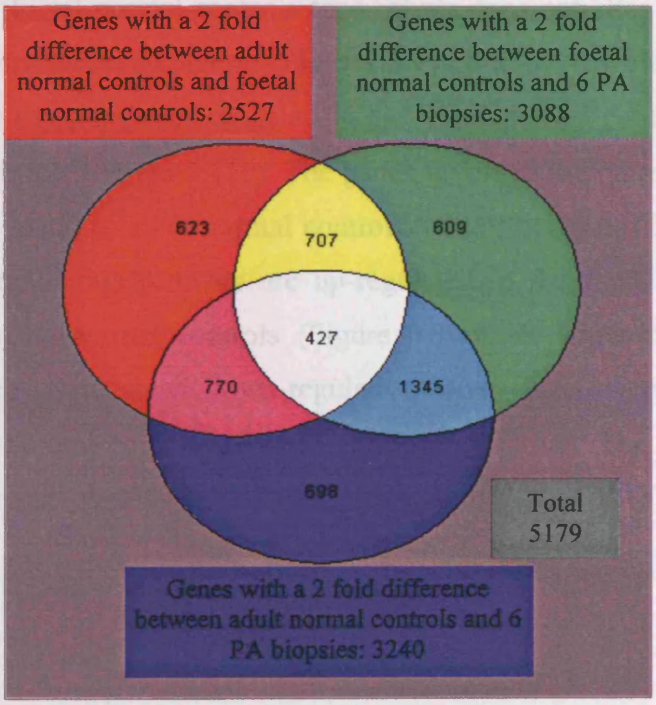
Figure 3.9 The dendrogram illustrates the expression profile similarity between the investigated controls, six paired paediatric pilocytic astrocytoma biopsies and short-term cell cultures and three paired adult glioblastoma multiforme biopsies and short-term cell cultures.



The dendrogram is coloured by 5304 genes that were present in all samples and used for this unsupervised hierarchical clustering approach. The samples with red branches are biopsy samples or controls, those with yellow branches are short-term cell culture samples or controls. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median. The abbreviations in the diagram are as follows; Cer = cerebellum, Cor Cal = corpus callosum, TB = total brain, NHA = normal human astrocytes. Tumour samples highlighted purple are paediatric PAI. Tumour samples highlighted green are adult GBMIV.

In total, 1345 probe pairs were identified that showed common differential expression in the PAI when either the foetal or adult normal controls were used (turquoise segment of the Venn diagram; representing 44% and 41% of the probe pairs when the foetal normal controls and adult normal controls were used respectively). In addition, probe sets that showed differential gene expression in the PAI when using the adult or foetal normal controls that were not identified when using the opposing control, did show a similar expression trend but did not show a greater than 2-fold change (the dark blue and green segments of the Venn diagram representing 698 (22%) and 609 (20%) probe sets respectively). A number of probe sets showed differential gene expression in PAI when using a specific control that did not show any expression alterations when using the opposing control (the pink (adult controls) and yellow (foetal controls) segments of the Venn diagram representing 770 (24%) and 707 (23%) probe sets respectively). These probe sets also showed differential gene expression between the adult and foetal normal controls.

Figure 3.10 Genes that show a 2-fold change in expression between the adult normal controls, the foetal normal controls and the six pilocytic astrocytoma biopsies are correlated in this Venn diagram.



The impact on cellular pathways of differentially expressed genes between PAI and specific controls was assessed using the Onto-tools software. Table 3.4 shows the pathway rankings for each comparison. 53 pathways were included in this analysis but

only those of interest are shown in the table (a list of all pathways in this approach can be found on the Supplementary Data CD included with this thesis). Overall pathway rankings are similar when using either foetal or adult normal controls. Moreover, of the top ten ranked KEGG pathways using either control group, eight were the same. These included antigen processing and presentation, cell adhesion molecules, phosphatidylinositol signalling system, MAPK signalling pathway, ECM receptor interaction, focal adhesion, regulation of actin cytoskeleton and calcium signalling. Figures 3.11, 3.12, 3.13 and 3.14 illustrate the gene expression pattern for each comparison in antigen processing and presentation, colorectal cancer, the cell cycle and Wnt signalling respectively. These pathways were investigated specifically as antigen processing and presentation was ranked first using both controls. The colorectal cancer pathway is the only characterised KEGG pathway for a specific cancer involved in this analysis approach and it was felt that this would be of specific interest. Disruption of the cell cycle is well characterised in astrocytomas and control of Wnt signalling is involved in foetal development.

The use of adult or foetal normal controls for analysis does not alter the results found for antigen processing and presentation (Figures 3.11a and 3.11b). However the genes of the MHC II pathway are down-regulated in the foetal normal controls compared to the adult normal controls (Figure 3.11c). The genes involved in colorectal cancer show up-regulation when foetal or adult normal controls are used (Figures 3.12a and 3.12b). Moreover, the genes of this pathway are up-regulated in the foetal normal controls compared to the adult normal controls (Figure 3.12c). A larger number of genes involved in the cell cycle show down-regulation when foetal normal controls are compared to adult normal controls (Figures 3.13a and 3.13b). The differential gene expression found in the Wnt signalling follows a similar pattern (Figures 3.14a and 3.14b).

Table 3.4 Comparison of KEGG pathways Onto-tool rankings between pilocytic astrocytoma biopsies and adult normal controls (green), pilocytic astrocytoma biopsies and foetal normal controls (blue) and foetal and adult normal controls (red). The genes used for this ranking approach showed a 2-fold and significant difference in expression between the selected groups (Figure 3.10). Of the 53 pathways investigated only those of interest are shown.

PAI biopsies compared to adult normal controls				PAI biopsies compared to foetal normal controls				Foetal normal controls compared to adult normal controls			
Rank	Pathway Name	Impact Factor	%Pathway Genes in Input	Rank	Pathway Name	Impact Factor	%Pathway Genes in Input	Rank	Pathway Name	Impact Factor	%Pathway Genes in Input
1	Antigen processing and presentation	112.08	26.7	1	Antigen processing and presentation	734.27	30.2	1	Antigen processing and presentation	119.33	14.0
2	Cell adhesion molecules	24.04	32.6	2	Cell adhesion molecules	31.93	35.6	2	ECM-receptor interaction	13.57	31.0
3	Phosphatidylinositol signalling system	19.97	30.4	5	Calcium signalling pathway	10.95	28.4	3	Phosphatidylinositol signalling system	12.18	24.1
4	MAPK signalling pathway	13.78	28.9	6	MAPK signalling pathway	9.46	25.3	4	Focal adhesion	10.54	24.2
6	ECM-receptor interaction	12.32	36.8	8	Phosphatidylinositol signalling system	9.35	27.8	5	Axon guidance	10.53	25.4
8	Focal adhesion	11.03	29.9	9	ECM-receptor interaction	9.19	32.2	6	Wnt signalling pathway	8.47	23.1
9	Regulation of actin cytoskeleton	9.22	27.2	10	Gap junction	8.60	31.3	7	Cell adhesion molecules	8.04	22.7
11	Calcium signalling pathway	8.35	26.1	11	Focal adhesion	8.60	26.8	8	Gap junction	7.20	25.3
14	Toll-like receptor signalling pathway	7.56	30.8	12	Regulation of actin cytoskeleton	7.04	24.3	10	Colorectal cancer	5.54	24.7
15	Neurodegenerative disorders	6.84	40.0	13	Apoptosis	6.71	29.8	13	Tight junction	5.09	21.0
16	Apoptosis	6.35	29.8	15	Toll-like receptor signalling pathway	5.65	26.4	14	Regulation of actin cytoskeleton	4.53	18.0
20	Gap junction	5.41	27.3	16	Natural killer cell mediated cytotoxicity	5.55	24.2	16	B cell receptor signalling pathway	3.98	17.5
21	B cell receptor signalling pathway	5.32	30.2	20	B cell receptor signalling pathway	4.61	22.2	19	Insulin signalling pathway	3.82	19.3
23	Adherens junction	5.16	29.9	23	Cytokine-cytokine receptor interaction	4.14	19.9	23	MAPK signalling pathway	3.40	16.5
28	Natural killer cell mediated cytotoxicity	4.13	23.4	25	Neurodegenerative disorders	3.96	31.4	26	Calcium signalling pathway	2.96	16.5

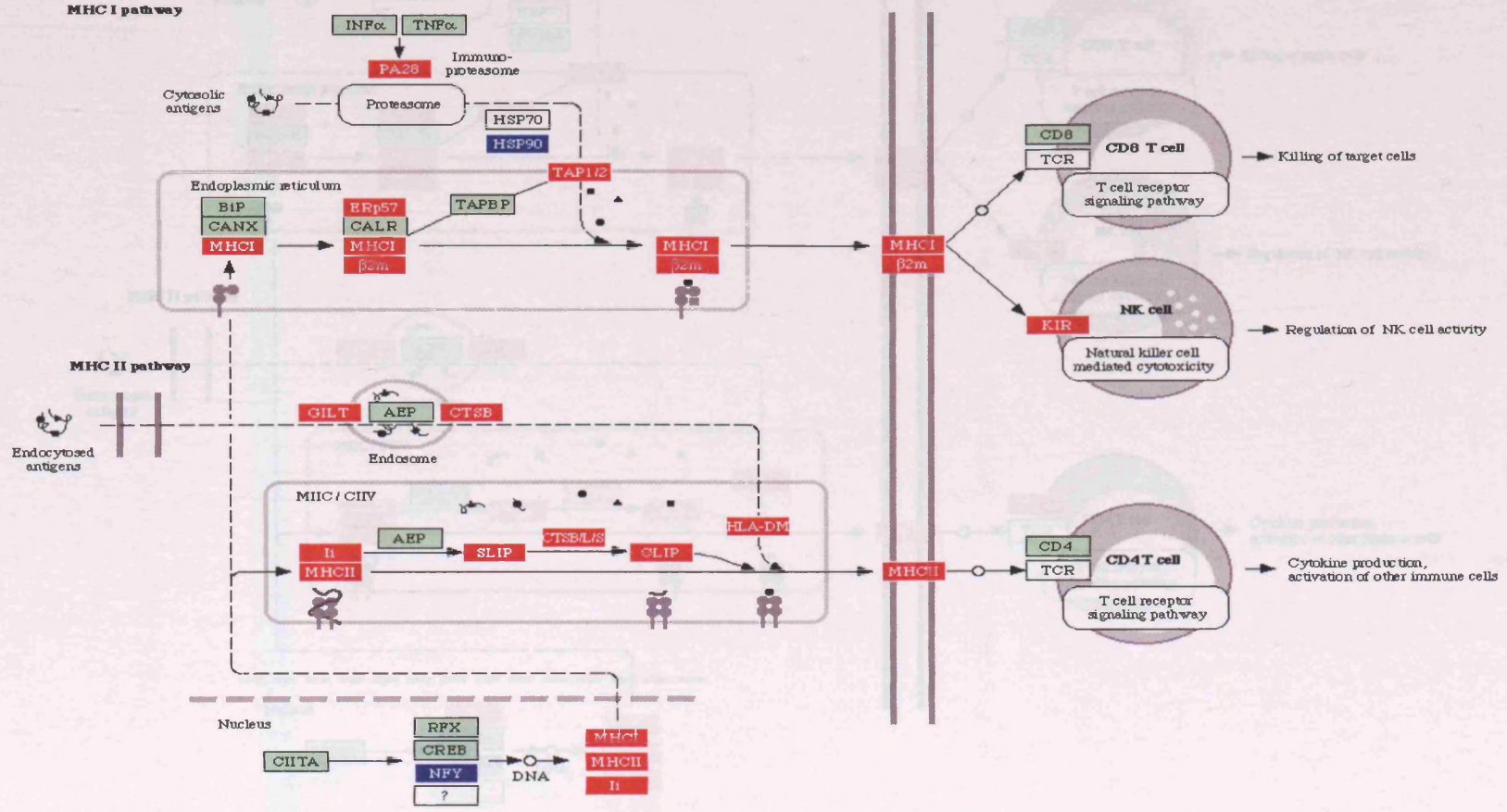
30	Neuroactive ligand-receptor interaction	3.97	18.9	26	Cell cycle	3.87	23.2	27	TGF-beta signalling pathway	2.79	14.3
32	Colorectal cancer	3.71	24.7	30	Adherens junction	2.86	23.4	30	Hedgehog signalling pathway	2.60	15.8
33	Cell cycle	3.62	23.2	31	Colorectal cancer	2.71	22.1	32	Adherens junction	2.43	18.2
34	T cell receptor signalling pathway	3.55	24.7	32	Neuroactive ligand-receptor interaction	2.47	17.2	36	Cell cycle	2.16	14.3
35	TGF-beta signalling pathway	3.37	23.8	33	VEGF signalling pathway	2.45	18.1	39	Apoptosis	1.91	14.3
36	Tight junction	3.05	21.0	35	Jak-STAT signalling pathway	2.09	16.3	40	VEGF signalling pathway	1.88	13.9
37	Wnt signalling pathway	2.99	20.4	39	T cell receptor signalling pathway	1.77	17.2	42	Neuroactive ligand-receptor interaction	1.79	9.6
38	VEGF signalling pathway	2.72	20.8	42	Tight junction	1.68	15.1	43	Natural killer cell mediated cytotoxicity	1.67	12.5
41	Cytokine-cytokine receptor interaction	2.45	18.4	43	Hedgehog signalling pathway	1.55	15.8	44	T cell receptor signalling pathway	1.67	14.0
42	Jak-STAT signalling pathway	2.35	18.3	44	SNARE interactions in vesicular transport	1.48	16.7	45	mTOR signalling pathway	1.60	14.3
47	SNARE interactions in vesicular transport	1.71	19.4	45	Wnt signalling pathway	1.43	15.6	46	Notch signalling pathway	1.55	13.0
48	Insulin signalling pathway	1.38	17.8	48	TGF-beta signalling pathway	1.23	14.3	47	Regulation of autophagy	1.38	10.3
49	mTOR signalling pathway	1.19	16.3	49	Notch signalling pathway	1.18	15.2	48	Jak-STAT signalling pathway	1.19	9.8
51	Hedgehog signalling pathway	0.95	7.0	50	Insulin signalling pathway	0.95	14.8	49	Toll-like receptor signalling pathway	1.12	9.9
52	Notch signalling pathway	0.71	10.9	52	mTOR signalling pathway	0.67	10.2	50	Cytokine-cytokine receptor interaction	1.09	6.6
53	Regulation of autophagy	0.49	6.9	53	Regulation of autophagy	0.60	3.4	51	SNARE interactions in vesicular transport	1.03	8.3

The 4 adult normal total brain samples showed a very high degree of expression profile similarity, as seen by in the tight clustering of these samples in the dendrogram of Figure 3.9. Moreover, the adult cerebellum and corpus callosum samples clustered with the foetal total brain, and showed variations between expression profiles. Only samples from these brain regions were available for investigation and although some tumours involved in this study are located in these regions, others are located in regions aside from the cerebellum or corpus callosum. Consequently adult total brain samples were used as normal controls for further analysis.

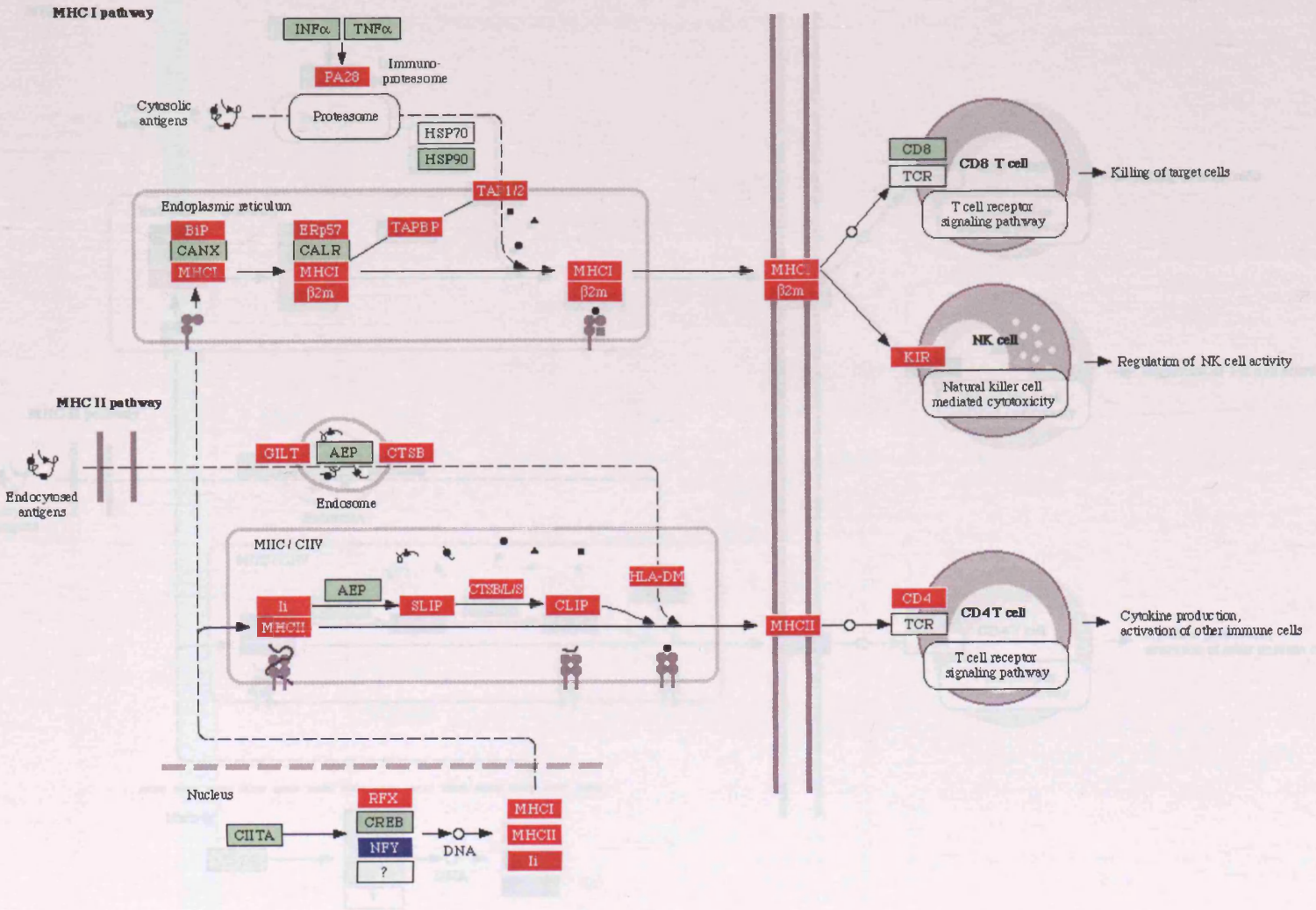
As a comparison between the biopsy and short-term cell culture experiments, those differentially expressed genes between the tumour and control samples were compared. From the 3240 differentially expressed probe sets between the 4 adult normal total brain controls and the 6 paediatric PAI biopsies and the 1695 differentially expressed probe sets between the 2 foetal astrocyte controls and the 6 paediatric PAI short-term cell cultures, only 556 probe sets were common to both analyses.

Figure 3.11 This Figure illustrates differential genes expression found in the KEGG pathway antigen processing and presentation. Genes shown in blue are down-regulated, genes shown in red are up-regulated and those genes in green are either normally expressed or no reliable data was available (those in white were not included in this approach).

a) Pilocytic astrocytoma biopsies compared to adult normal controls.



b) Pilocytic astrocytoma biopsies compared to foetal normal controls.



c) Foetal normal controls compared to adult normal controls.

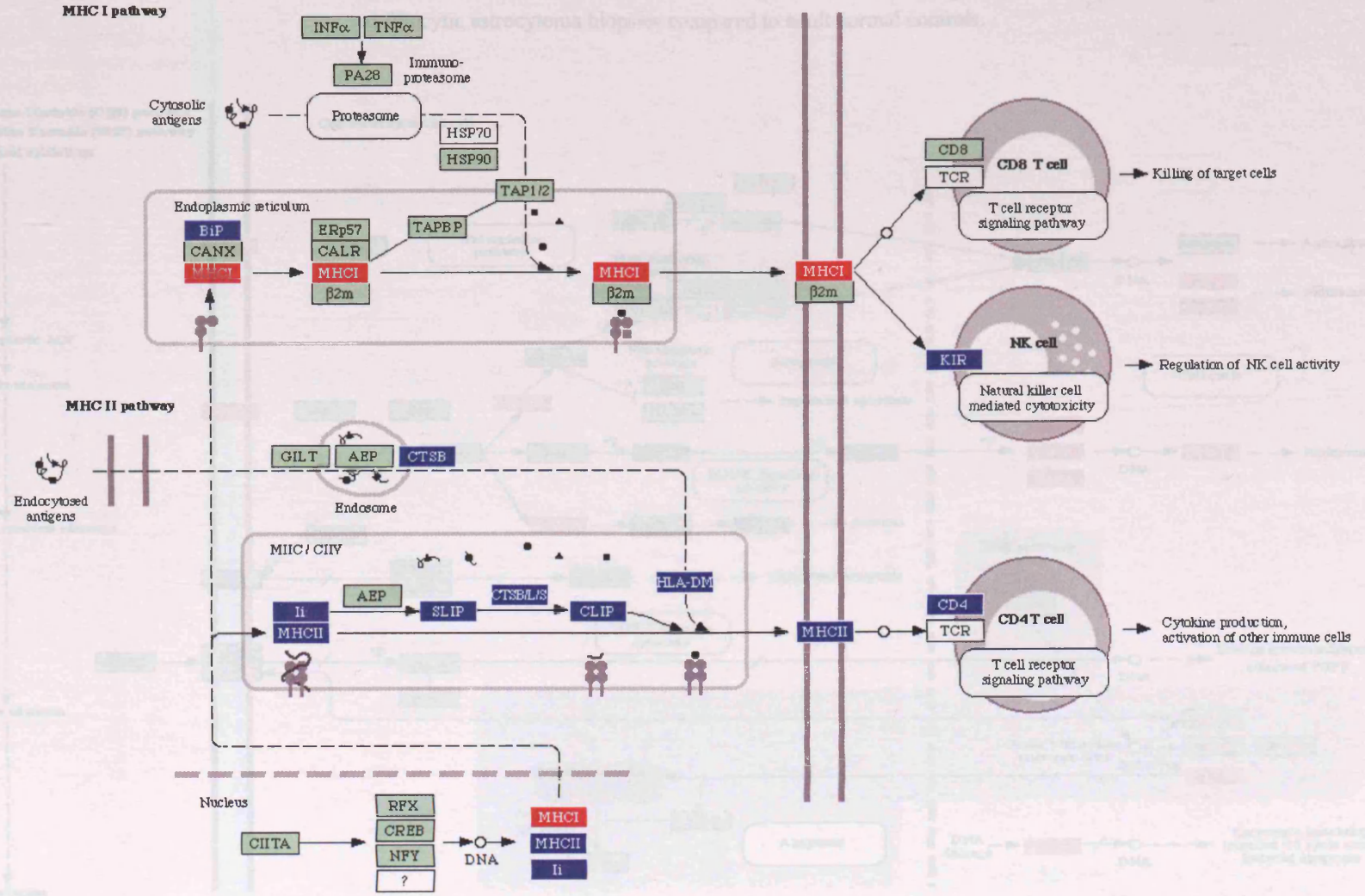
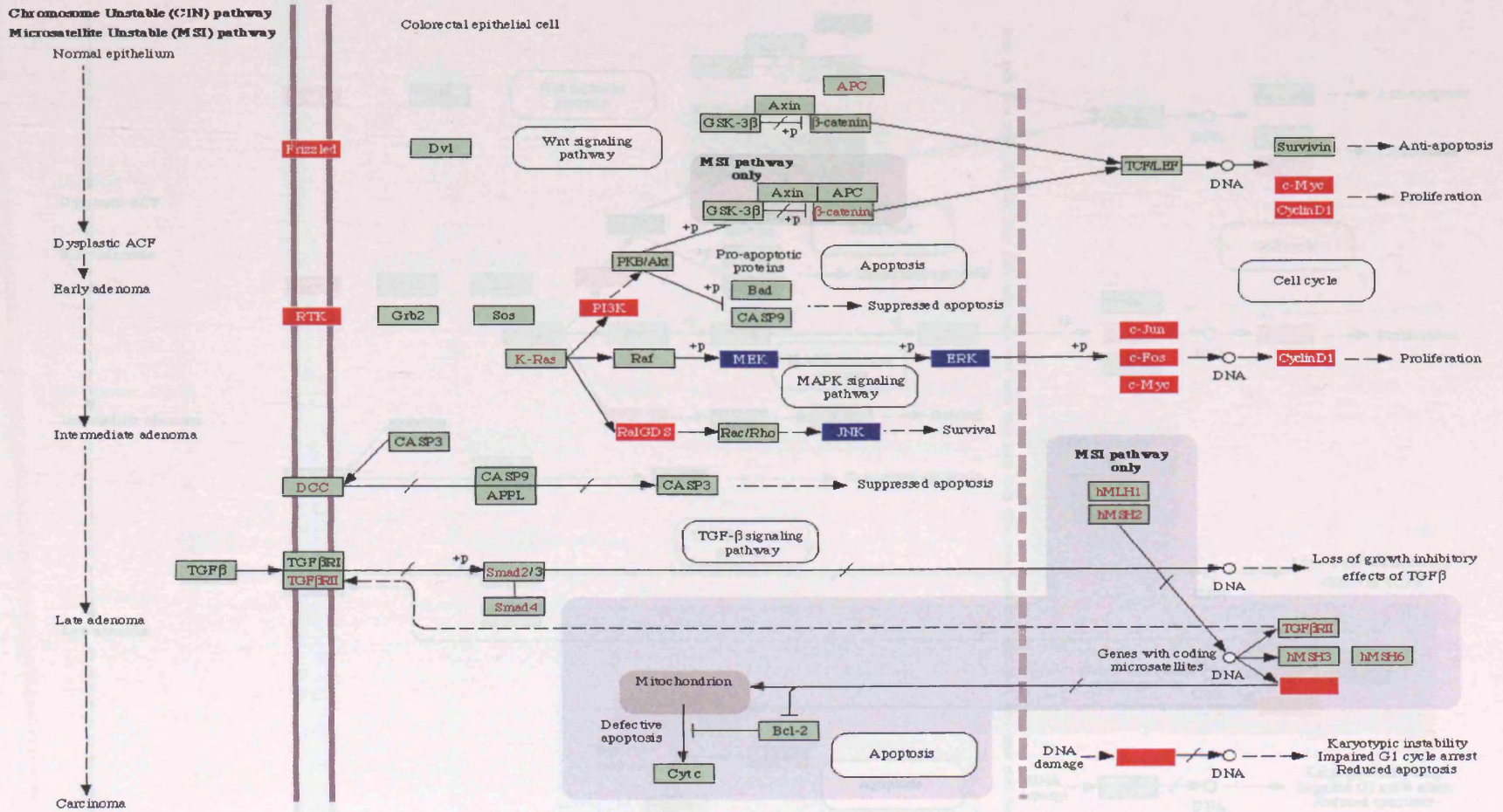
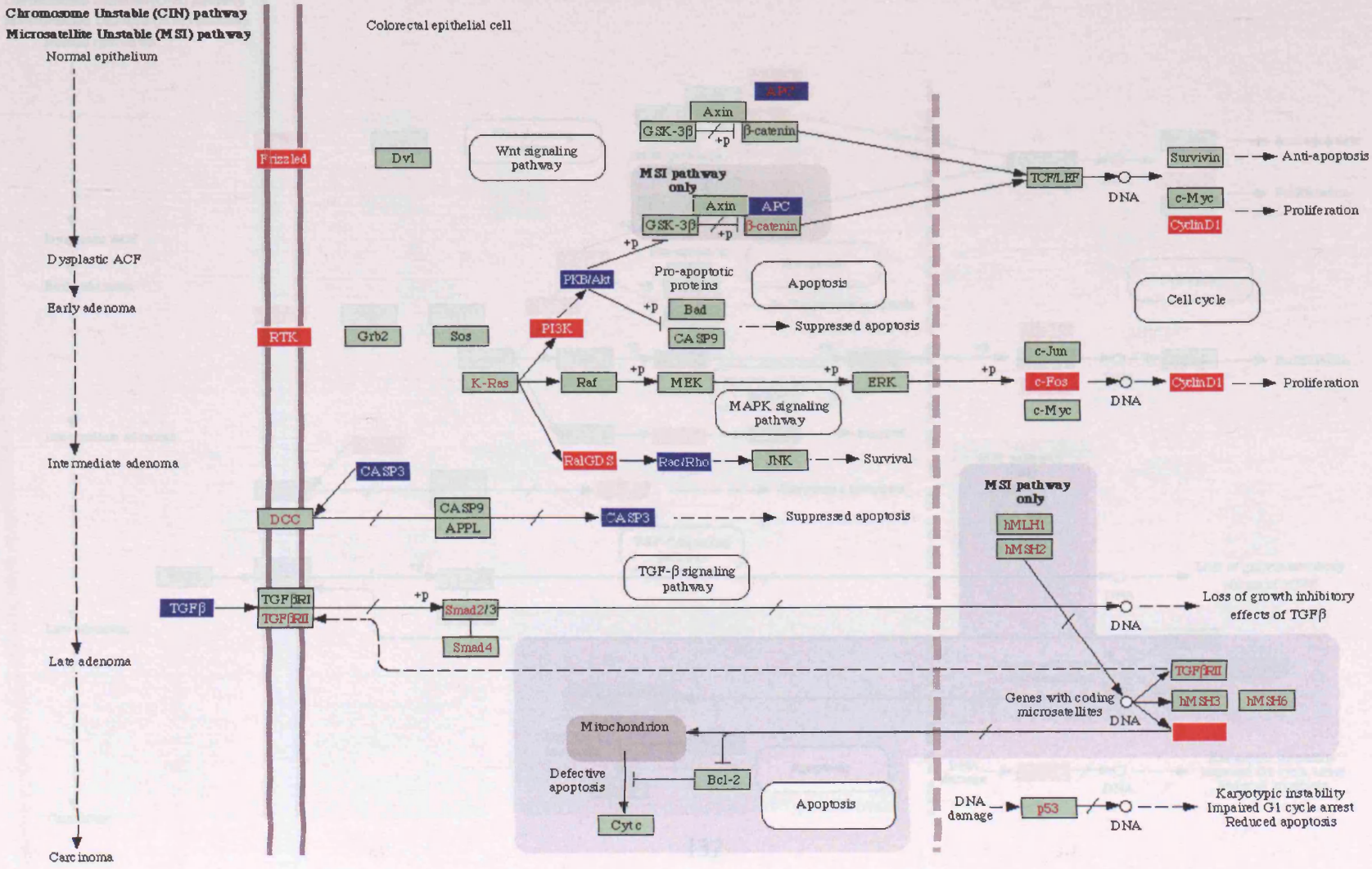


Figure 3.12 This Figure illustrates differential genes expression found in the KEGG pathway colorectal cancer. Genes shown in blue are down-regulated, genes shown in red are up-regulated and those genes in green are either normally expressed or no reliable data was available (those in white were not included in this approach).

a) Pilocytic astrocytoma biopsies compared to adult normal controls.



b) Pilocytic astrocytoma biopsies compared to foetal normal controls.



c) Foetal normal controls compared to adult normal controls.

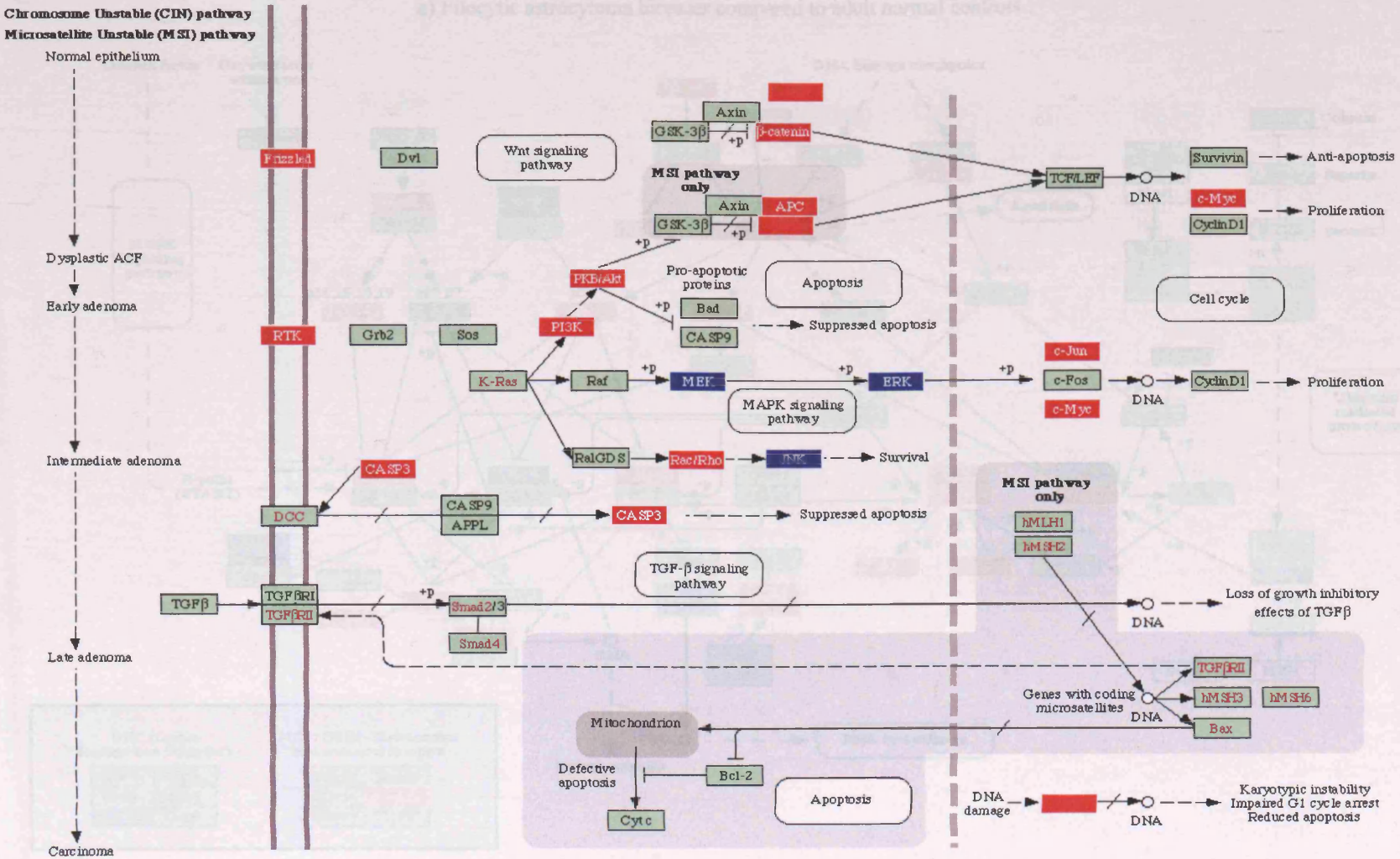
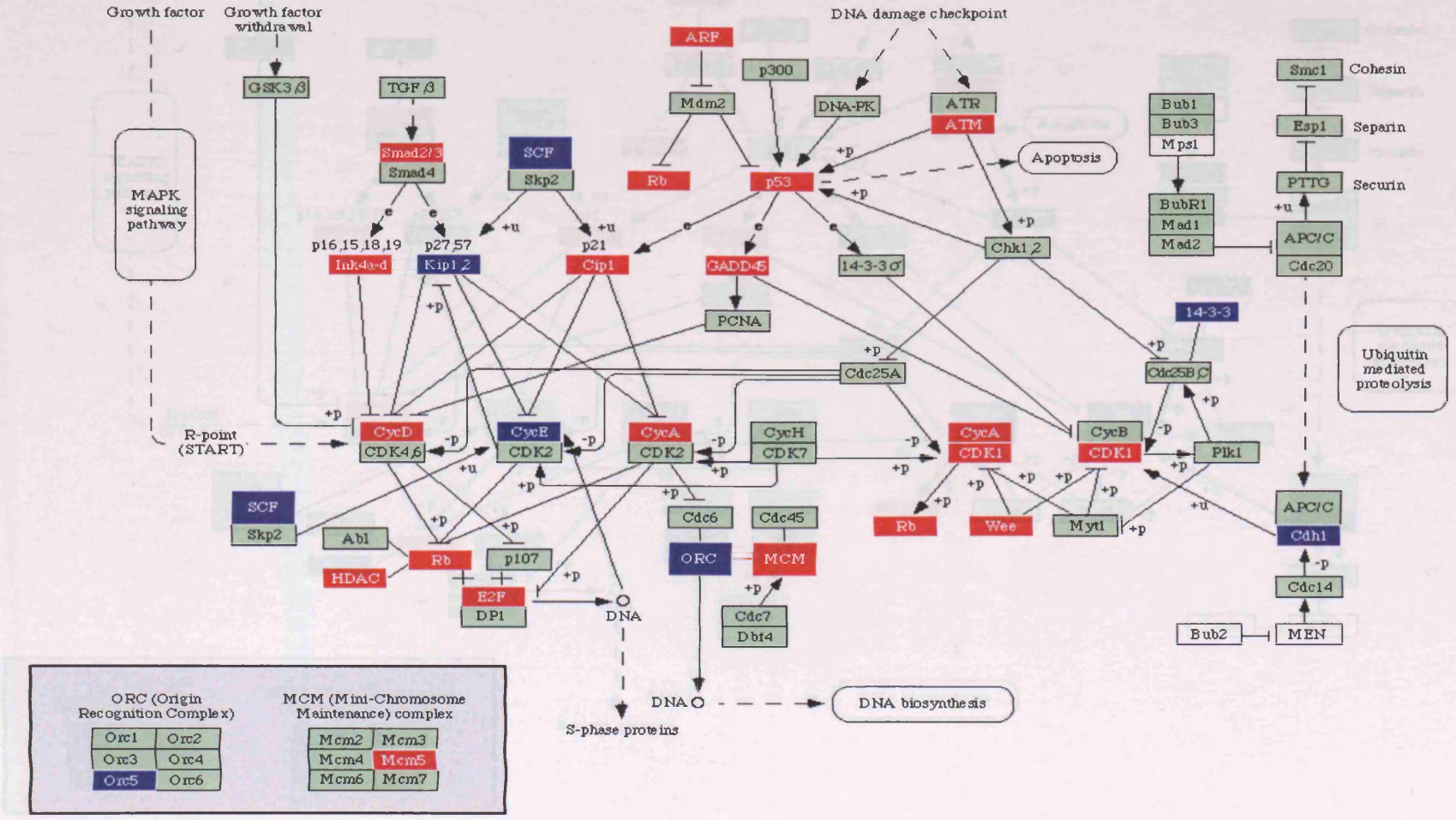
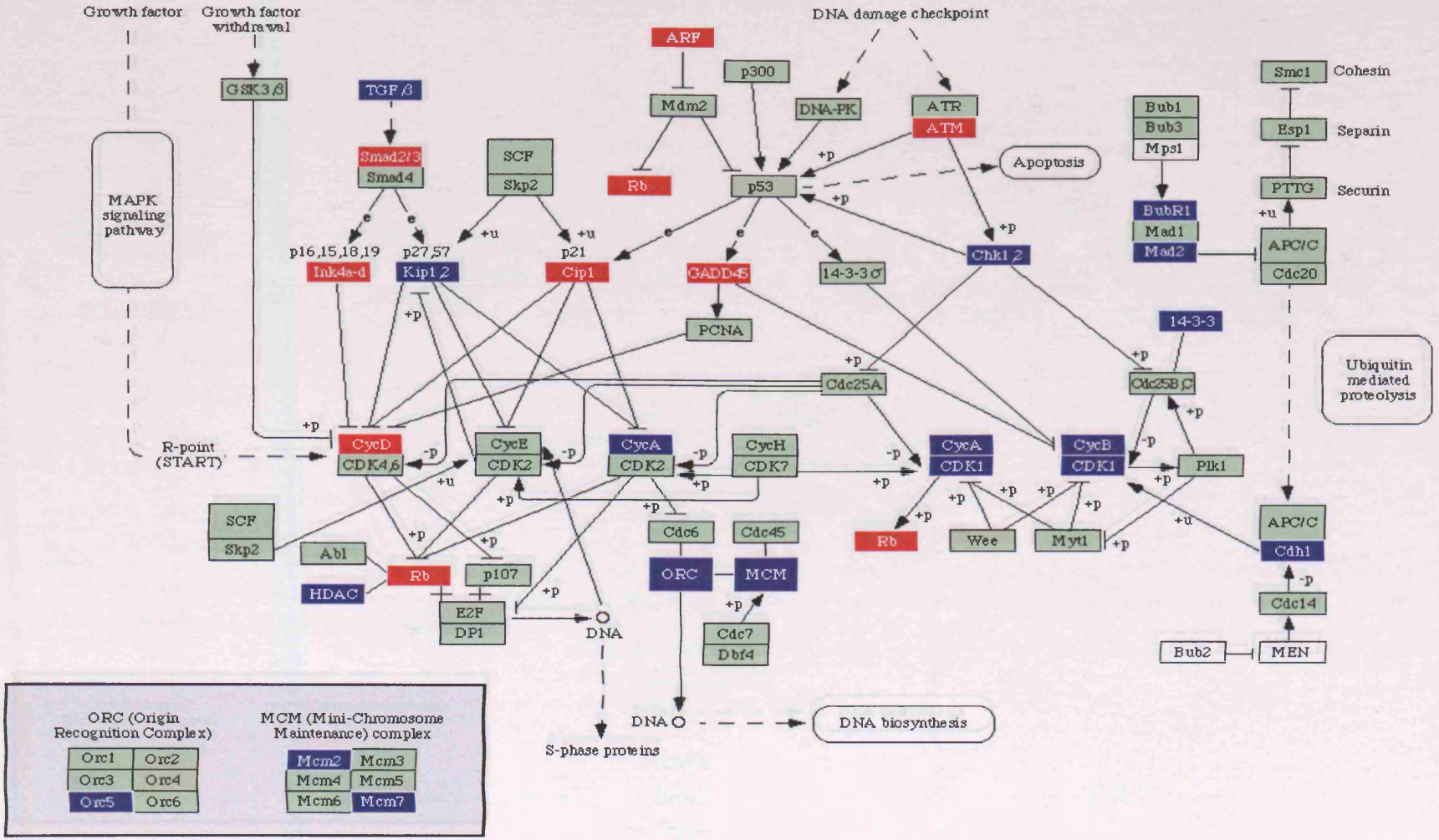


Figure 3.13 This Figure illustrates differential genes expression found in the KEGG pathway cell cycle. Genes shown in blue are down-regulated, genes shown in red are up-regulated and those genes in green are either normally expressed or no reliable data was available (those in white were not included in this approach).

a) Pilocytic astrocytoma biopsies compared to adult normal controls.



b) Pilocytic astrocytoma biopsies compared to foetal normal controls.



c) Foetal normal controls compared to adult normal controls.

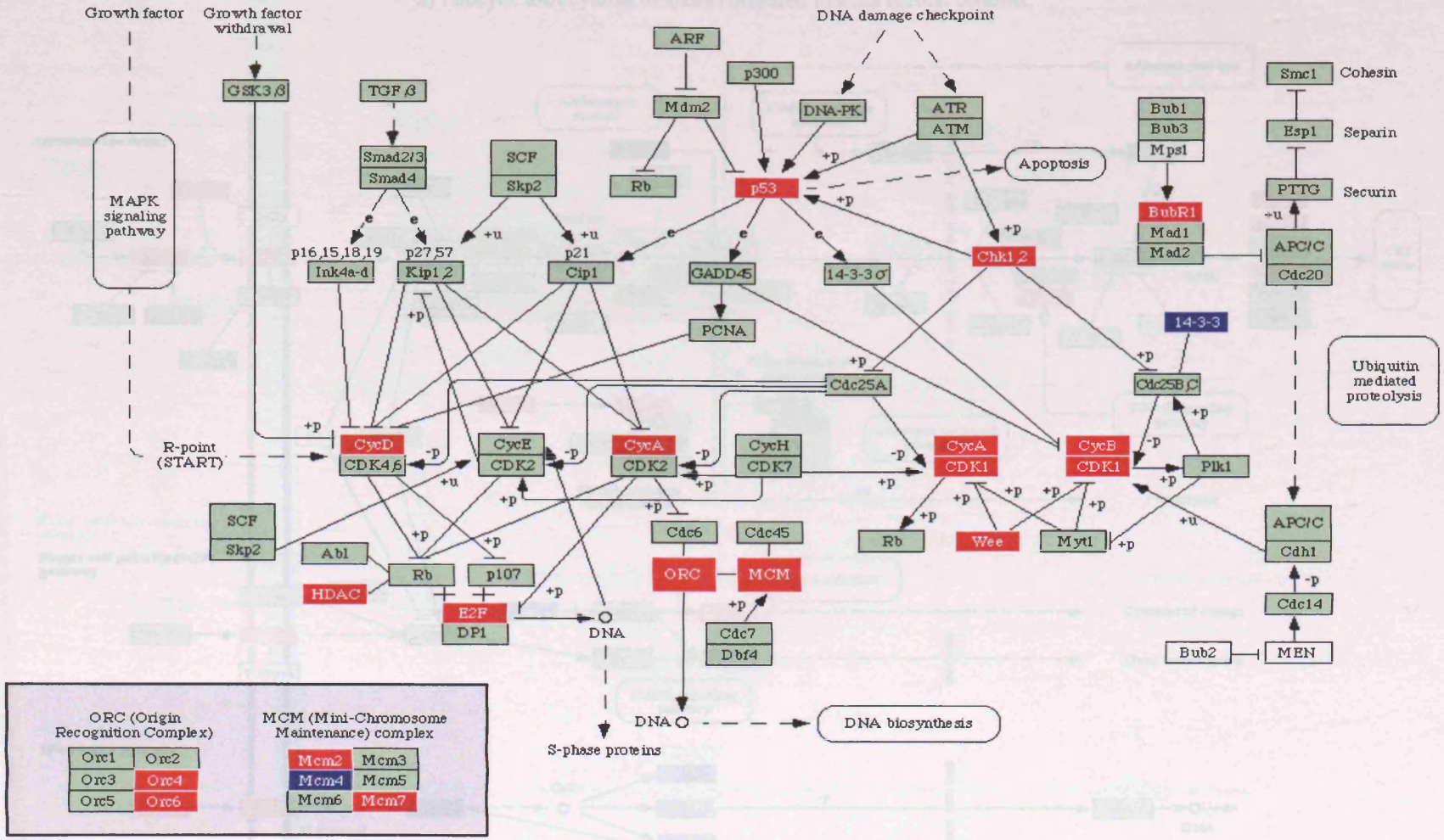
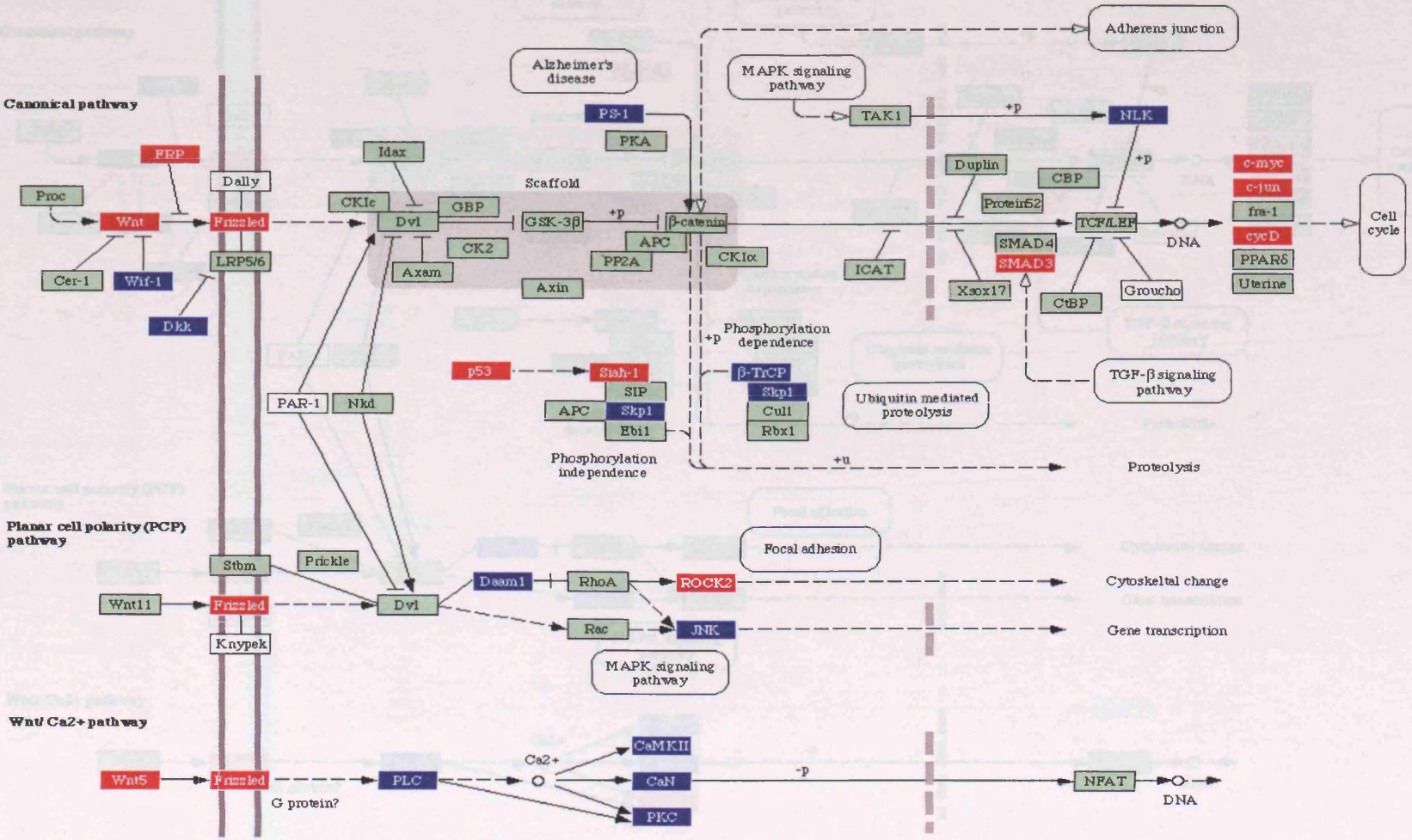
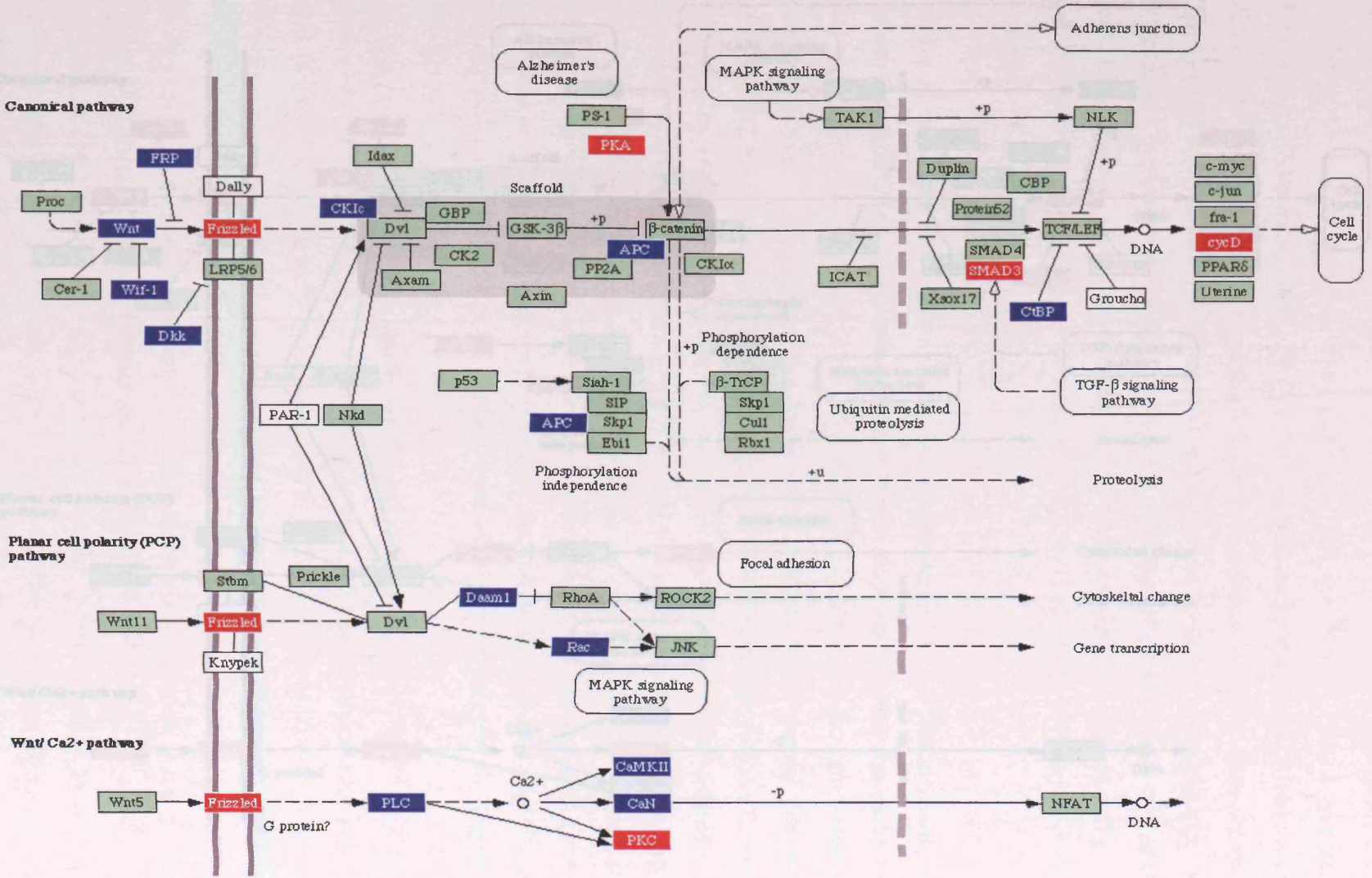


Figure 3.14 This Figure illustrates differential genes expression found in the KEGG pathway Wnt signalling. Genes shown in blue are down-regulated, genes shown in red are up-regulated and those genes in green are either normally expressed or no reliable data was available (those in white were not included in this approach).

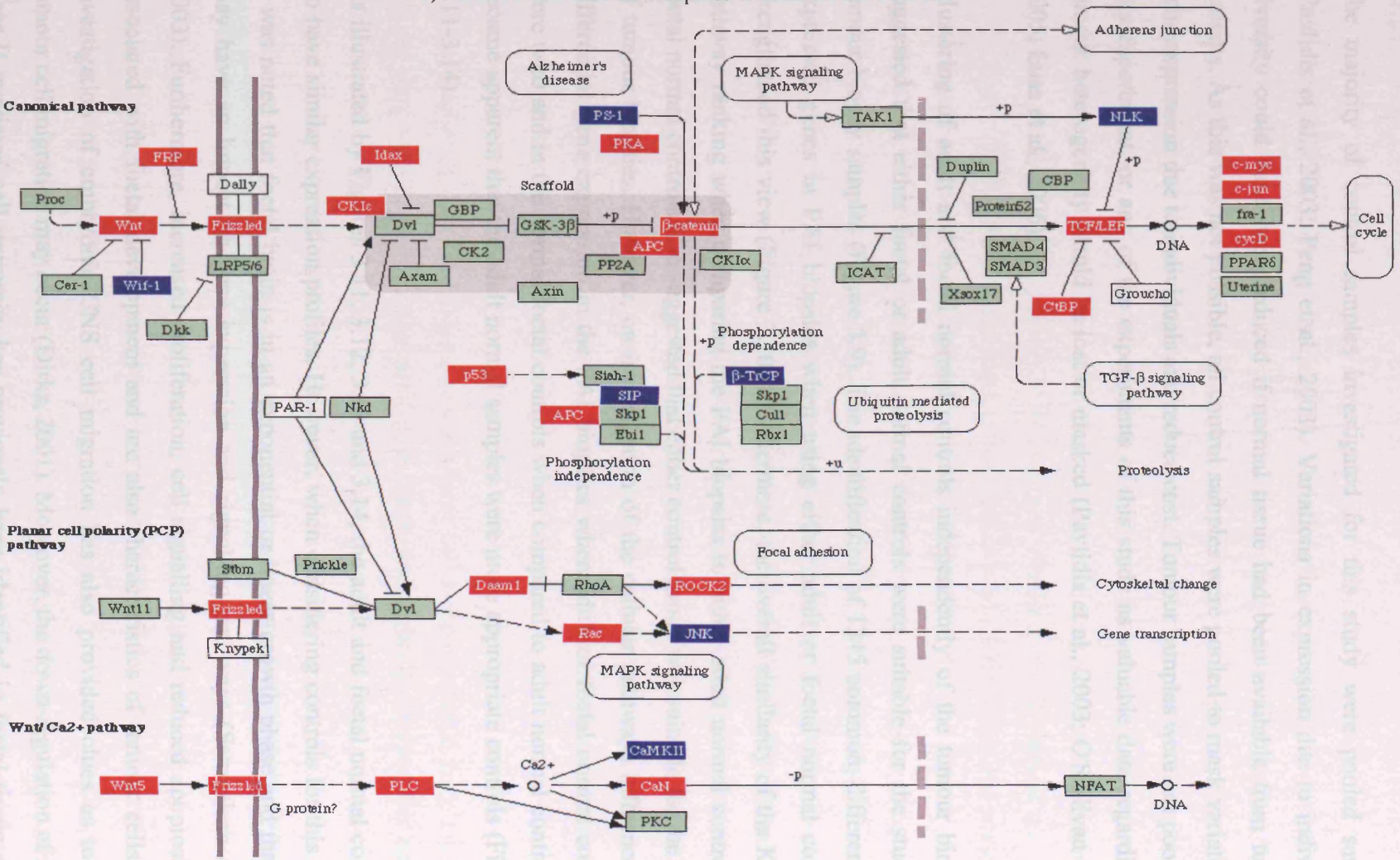
a) Pilocytic astrocytoma biopsies compared to adult normal controls.



b) Pilocytic astrocytoma biopsies compared to foetal normal controls.



c) Foetal normal controls compared to adult normal controls.



DISCUSSION

The majority of control samples investigated for this study were pooled samples (Pavlidis et al., 2003; Peng et al., 2003). Variations in expression due to individual diversity could have been reduced if normal tissue had been available from tumour patients. As this was not possible, all control samples were pooled to mask variation in gene expression due to individuals and reduce cost. Tumour samples were not pooled in this experiment, or any of the experiments of this study as valuable data regarding to tumour heterogeneity would be lost or masked (Pavlidis et al., 2003; O'Sullivan et al., 2005; Lusa et al., 2006).

Clustering of adult and foetal normal controls independently of the tumour biopsies suggested that either foetal or adult normal controls were suitable for the study of tumour biopsy samples (Figure 3.9). The identification of 1345 common, differentially expressed genes in PAI biopsies when using either adult or foetal normal controls strengthened this view (Figure 3.10). Furthermore, the overall similarity of the KEGG pathway ranking when comparing the PAI biopsies to either adult normal controls or foetal normal controls also suggested that either control group was suitable for the study of tumour biopsies. However, on investigation of the cellular pathways influenced by differential gene expression in the PAI biopsies when adult or foetal normal controls were used and in the normal foetal controls when compared to adult normal controls, it became apparent that the adult normal samples were more appropriate controls (Figures 3.11-3.14).

As illustrated by Figures 3.11, 3.12, 3.13 and 3.14, the adult and foetal normal controls do have similar expression profiles. However, when considering controls for this study it was noted that foetal brain is in an exponential or linear growth phase and that this may have an impact on gene expression and signalling pathways (Samuelsen et al., 2003). Furthermore, increased proliferation, cell signalling and reduced apoptosis are associated with foetal development and are also characteristics of tumour cells. The investigation of embryonic CNS cell migration has also provided clues as to how tumour cell migration may occur (Dirks, 2001). Moreover, the down-regulation of MHC class II mediated cell immunity has previously been identified in foetal tissue and is

thought to be linked to a high success rate when transplanting foetal tissue (Bhattacharya, 2004).

The genes of the Wnt signalling pathway and cell cycle are clearly up-regulated in the foetal normal controls compared to the adult normal controls. It was hypothesised that the gene expression profile of foetal normal controls may mask aspects of the tumour profile when used as controls. This was seen in the tumour expression profile of the Wnt signalling pathway, the cell cycle and to a lesser extent the colorectal cancer pathway when foetal normal controls were used. Moreover, the Wnt signalling pathway has been implicated in foetal development (McMahon and Bradley, 1990; Parr et al., 1993; Hoang et al., 1998; Smalley and Dale, 1999; Katoh et al., 2000). For this reason the adult normal controls were chosen as controls for this study.

In addition, it was also decided to use only the adult total brain samples as controls. Significant variation was seen between the expression profiles of total brain, cerebellum and corpus callosum from both adult and foetal origin. Furthermore, the tumours of this study are located in brain regions aside from the cerebellum and corpus callosum and it was felt inappropriate to compare tumour samples from different locations to normal controls taken from these regions. Consequently, to standardise the normal control group and reduce sample heterogeneity, four pooled total brain samples from between 3 and 5 individuals were chosen as controls.

The expression profiles of biopsy and short-term cell culture samples from the same tumour show variations when compared. This is not unexpected given sampling and cell culture conditions as previously discussed. Furthermore, the biopsy and cell culture samples clearly cluster into 2 groups regardless of sample origin, highlighting cell culture as the most important parameter in this experiment. Consequently, it was not possible to use normal brain samples as the control for tumour short-term cell culture experiments. Astrocytes are the proposed cell of origin of astrocytomas and the tumours are often positive for astrocytic markers. Consequently, foetal astrocytes were chosen as normal controls for the tumour short-term cell cultures of this study. Adult astrocytes were not available for additional comparisons and consequently no comparisons were possible when selecting this control. Due to a sample number of only 2, the foetal

astrocyte controls have been included in further experiments with caution and have not been directly used for experiment normalisations.

The dendrogram of Figure 3.9 illustrates the grouping of all biopsy and short-term cell culture samples separately, suggesting that regardless of sample type the most dominating sample parameter influencing gene expression is cell culture. This confirms the need for independent normal controls for each sample type. Furthermore, the comparison of those genes showing differential gene expression between tumour biopsies and adult total brain and tumour short-term cell cultures and foetal astrocytes highlights the differences between these sampling approaches and the influences this has on paediatric astrocytoma study.

Short-term cell cultures are a valuable tool in the study of astrocytoma and other tumours and cancer. They represent a resource that increases the availability of tumour RNA and DNA for study. Moreover, short-term cell cultures provide a means to investigate cell characteristics such as cell surface markers, proliferation rate, cell motility, chemoresistance and response to stimuli including growth factors, extracellular matrix components, proteases and adhesion molecules. The manipulation of gene expression through a variety of mechanisms including RNA interference, target antibodies and gene therapy allows investigators to study cell response. This provides a working platform to assess the efficacy of target therapies and validate further investigation in *in vivo* studies.

CHAPTER 4

DIFFERENTIAL GENE EXPRESSION AND COPY NUMBER ALTERATIONS IN PAEDIATRIC PILOCYTIC ASTROCYTOMA BIOPSIES

INTRODUCTION

PAI are the most common brain tumour in children (Louis et al., 2007a). Cytogenetic analysis of paediatric PAI has shown that the majority of these tumours (>70%) have a normal karyotype (Bhattacharjee et al., 1997). Numerical and structural abnormalities of chromosomes 5, 6, 7, 8 and 9 have been reported, although definitive, non-random aberrations have not yet been identified (White et al., 1995; Zattara-Cannoni et al., 1998; Shlomit et al., 2000; Sanoudou et al., 2000b; Rickert and Paulus, 2004). It has been suggested that epigenetic events in low-grade astrocytoma such as aberrant promoter hypermethylation are more common than genetic alterations and that they may have a greater impact on tumour development (Costello et al., 2000).

Few individual studies have focused on differentially expressed genes in paediatric PAI. However, two microarray studies have investigated paediatric PAI. The study by Wong et al. (2005) analysed 21 paediatric PAI using the Affymetrix GeneChip® U133A array. A second study focused on 41 PAI ranging in age between 1 and 53 years which were a mixture of sporadic PAI and those arising in patients with NF1 (Sharma et al., 2007). These studies have previously been discussed in detail in Chapter 1.

The aim of this chapter was to identify differential gene expression in paediatric PAI biopsies compared to 4 adult normal total brain controls and investigate genomic CNAs and methylation as a mechanism for these alterations to characterise disrupted cellular pathways that could be involved in tumour development and growth. The Affymetrix GeneChip® U133A array validated by Q-PCR was used to generate gene expression profiles and identify aberrantly expressed genes in 19 paediatric PAI biopsies and 4 normal adult total brain controls. Six of these paediatric PAI biopsies are the same samples as those used in Chapter 3 including IN1740, IN2110, IN2356, IN2368, IN2921 and IN3013. The aCGH SpectralChip™ 2600 array was also used to define genomic aberrations in 11 of the 19 paediatric PAI biopsies. Limited tumour biopsy

material was available for this study, consequently priority was given to RNA extraction and expression analysis as it was felt that this would prove to be the most significant in the study of PA

To further investigate mechanisms that may induce transcriptional silencing in PAI, MSP was used to investigate the promoter region methylation status of six genes comprising *CDKN1C, PRDM2, SPINT2, REPRIMO, CCNA1* and *DAPK1*, that showed a 2-fold and significant down-regulation in the PAI compared to the normal controls (Olek et al., 1996b; Warnecke et al., 1997; Grunau et al., 2001). As well as showing down-regulation in the PAI, these genes had previously been reported as being methylated in astrocytoma or other tumours or cancer. Furthermore, hypermethylation of these 6 genes has been detected in more than 30% of several tumours types including gastric carcinoma, lung cancer and renal cell carcinoma Moreover, *CCNA1* was hypermethylated in 100% of colorectal adenoma (Kikuchi et al., 2002; Toyooka et al., 2003; Xu et al., 2004; Oshimo et al., 2004; Morris et al., 2005; Takahashi et al., 2005). Only one previous study has investigated hypermethylation in paediatric PAI involving *MGMT, GSTP1, DAPK1, p14^{ARF}, THBS-1, TIMP-3, p73, CDKN2A, RB-1* and *TP53*. Hypermethylation of *CDKN2A* was most frequent, occurring in 46% of cases. However, 5 of the remaining genes were hypermethylated in less than 8% of cases (Gonzalez-Gomez et al., 2003b). In total, 17 PAI were included as sufficient DNA was not available from the 2 remaining tumours.

PAI tumour biopsy tissue was available from 19 paediatric patients, details of which can be found in Table 2.2. The mean patient age at diagnosis was 5.8 years, (range, 1.75 to 11.5 years) with a female/ male ratio of 8/11. At the time of this study patient survival ranged from 1-160 months from diagnosis. The initial diagnosis of all 19 tumours in this Chapter remained the same according to the 2007 WHO grading schemes.

The experiment analysis was carried out as previously described and the mean expression of each gene in all samples was normalised to that of the control samples for the per gene normalisation. The control samples used here were 4 adult normal total brain controls, details of these samples and their use as a control is documented in Chapter 3. Normalising to the control samples centres the experiment about the average expression level of each gene in the control samples. Gene probe sets present in 19 of

the 23 samples (10554) were included in the experimental analysis as well as probe sets that were called as present in all 4 normal controls but absent in all 19 PAI (99). This allowed those genes that were down-regulated in either sample group and consequently detected as absent on the array to be included in further analysis.

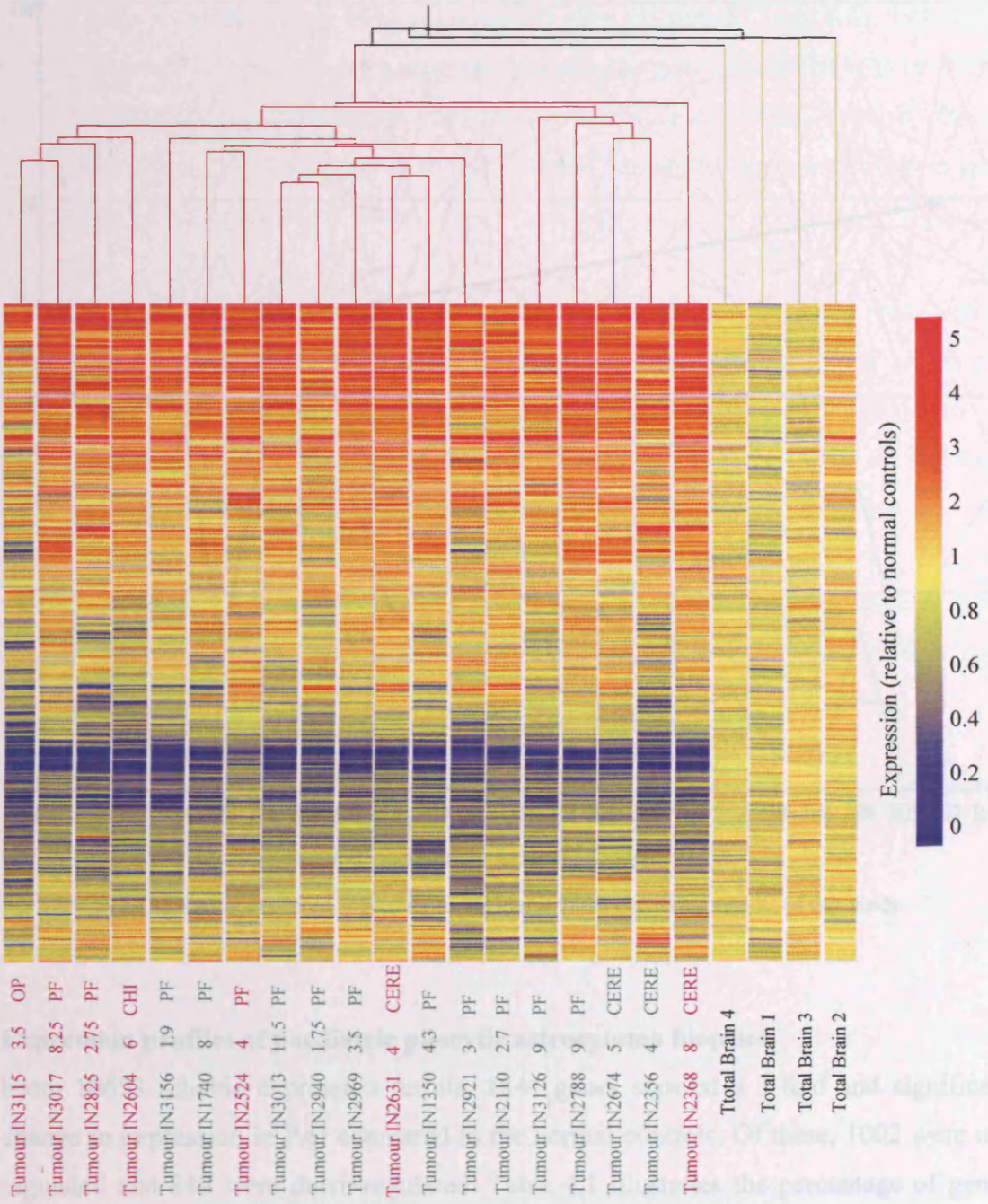
RESULTS

Paediatric pilocytic astrocytoma biopsy clustering, sub-groups and molecular signatures

Unsupervised hierarchical clustering using 10653 reliable gene expression results was used to generate a dendrogram illustrating the similarity in expression profiles of the 19 paediatric PAI biopsies compared to 4 adult normal brain controls (Figure 4.1). The tumours do not group or order according to patient age, sex or tumour location and no further sub-groups were clearly defined, contradictory to that previously shown by Wong et al., 2005.

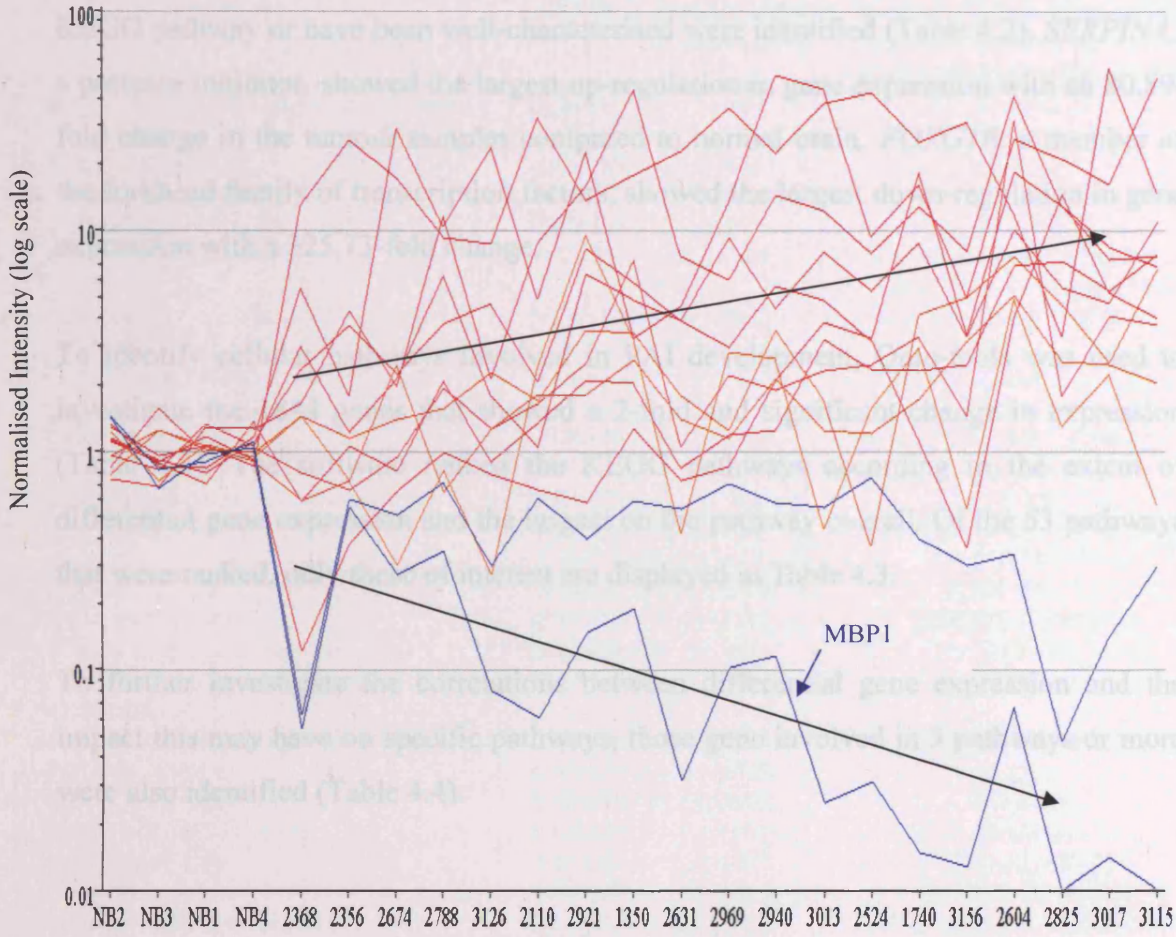
Wong et al. (2005) described a molecular signature of 89 genes that distinguished two subgroups of PAI representing tumours with different potentials of progression. A list of the 89 genes was not available. However, 16 of the 89 genes were described by Wong et al. (2005) and involved in biological processes significantly altered between the two sub-groups. These 16 genes were investigated in the PAI of this study. Only 13 of the 16 genes showed reliable data and were used to cluster the 19 PAI. The PAI did not cluster into sub-groups and Figure 4.2 illustrates a continuous increase or decrease in the expression of these 13 genes between tumours that are least similar.

Figure 4.1 Unsupervised hierarchical clustering of 19 paediatric pilocytic astrocytoma and 4 normal brain controls using 10653 reliable gene expression results.



The dendrogram colour saturation is proportional to the magnitude of the difference from the mean relative to controls, ranging from red (over-expressed) to blue (under-expressed). Patient ages are in years at diagnosis, tumour labels coloured green indicate a male patient and pink a female patient and location abbreviations are as follows: OP = optic pathway; PF = posterior fossa; CHI = chiasmatic; CERE = cerebellum.

Figure 4.2 The profile of 13 reliable expression results from this study, also used by Wong et al. (2005) to distinguish two possible sub-groups in paediatric pilocytic astrocytoma.



The 19 PAI are ordered according to the hierarchical clustering results of this study.

Expression profiles of paediatric pilocytic astrocytoma biopsies

From 10653 reliable expression results, 1844 genes showed a 2-fold and significant change in expression in PAI compared to the normal controls. Of these, 1002 were up-regulated and 842 were down-regulated. Table 4.1 illustrates the percentage of genes disrupted in specific Gene Ontology (GO) biological processes (Thomas et al., 2007). Approximately 5-10% of genes in each GO group showed differential gene expression. The cell homeostasis GO showed the highest number of altered genes (11%), compared to chemi-mechanical coupling which was the lowest (3%). The GO of those genes investigated using Q-PCR and MSP are also shown in Table 4.1.

In order to investigate those gene that were most differentially expressed, genes that showed a greater than 10-fold change in expression and were either involved in a KEGG pathway or have been well-characterised were identified (Table 4.2). *SERPINA3* a protease inhibitor, showed the largest up-regulation in gene expression with an 80.89-fold change in the tumour samples compared to normal brain. *FOXG1B*, a member of the forkhead family of transcription factors, showed the largest down-regulation in gene expression with a 325.73-fold change.

To identify cellular pathways involved in PAI development, Onto-tools was used to investigate the 1844 genes that showed a 2-fold and significant change in expression (Table 4.3). The software ranked the KEGG pathways according to the extent of differential gene expression and the impact on the pathway overall. Of the 53 pathways that were ranked, only those of interest are displayed in Table 4.3.

To further investigate the correlations between differential gene expression and the impact this may have on specific pathways, those gene involved in 5 pathways or more were also identified (Table 4.4).

Table 4.1 Gene ontologies of specific genes differentially expressed between pilocytic astrocytoma and normal controls.

Biological process	GO ID	Number of genes in GO group	Number of genes differentially expressed between paediatric astrocytoma and normal controls
Amino acid and derivative metabolism	0006519	180	13 (7%)
Behaviour	0007610	83	8 (10%)
Biosynthesis	0009058	885	52 (6%)
Carbohydrate metabolism	0005975	1696	134 (8%)
Catabolism	0009056	426	27 (6%)
Cell cycle	0007049	1161	78 (7%) (MAPK1, CDKN1C, CCNA1, MATK, REPRIMO)
Cell death	0008219	863	66 (8%) (MAPK1, DAPK1, MCL1)
Cell differentiation	0030154	1657	144 (9%) (TIMP1, MATK, MCL1)
Cell growth	0016049	3993	288 (10%) (TIMP1, CDKN1C, MATK, MCL1)
Cell homeostasis	0019725	90	10 (11%)
Cell proliferation	0008283	1067	82 (8%) (TIMP1, CDKN1C, MATK)
Cell recognition	0008037	1820	161 (9%)
Cell-cell signalling	0007267	2582	220 (9%) (SPINT2)
Chemi-mechanical coupling	0006943	102	3 (3%)
Co-enzyme and prosthetic group metabolism	0006731	1163	64 (6%)
Cytoskeleton organisation and biogenesis	0007010	1536	131 (9%) (MAPK1)
DNA metabolism	0006259	1470	75 (5%)
Electron transport	0006118	3286	210 (6%)
Embryonic development	0009790	779	44 (6%)
Energy pathways	0006091	1497	71 (5%) (DAPK1)
Growth	0040007	1405	123 (9%) (SPINT2)
Host-pathogen interactions	0030383	4051	301 (7%) (CCNA1, MATK)
Ion transport	0006811	2474	193 (8%)
Lipid metabolism	006629	859	82 (10%)
Mitochondria organisation and biogenesis	0007005	1273	66 (5%) (DAPK1)
Morphogenesis	0009653	301	11 (4%)
Protein biosynthesis	0006412	1060	53 (5%)
Protein modification	0006412	307	24 (8%)
Protein transport	0015031	2031	203 (10%)
Regulation of gene expression; epigenetic	0040029	3969	241 (6%) (TIMP1, CDKN1C, PRDM2, CCNA1, MATK)
Reproduction	0000003	141	8 (6%)
Response to endogenous stimuli	0009719	103	9 (8%)
Response to stress	0006950	427	31 (7%) (MAPK1, MCL1)
Signal transduction	0007165	4158	330 (8%) (MAPK1,DAPK1)
Transcription	0006350	3697	185 (5%) (PRDM2)
Unknown biological processes	0000004	11034	379 (3%)

The percentage of genes in each ontology group that shows differential expression between the PAI and normal controls is shown in brackets. The GO of those genes investigated using Q-PCR and MSP are indicated in column 4.

Table 4.2 Genes identified from the 1844 differentially expressed genes that show a greater than 10-fold change in expression and are involved in specific pathways or are well-characterised with functions that can be linked to tumour development.

Rank	Gene symbol	Gene description	Fold change in expression	Chromosome location
1	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	80.89	14q32.1
2	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	56.98	1q32.1
4	CHAD **2** (4,11)	chondroadherin	43.68	17q21.33
6	TIMP4	tissue inhibitor of metalloproteinase 4	41.47	3p25
7	WEE1 **1** (38)	WEE1 homolog (S. pombe)	41.03	11p15.3-p15.1
11	T1A-2	lung type-I cell membrane-associated glycoprotein	35.21	1p36
12	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	33.72	17q22
15	IL1RAP **2** (1,47)	interleukin 1 receptor accessory protein	26.58	3q28
17	CSPG4	chondroitin sulfate proteoglycan 4 (melanoma-associated)	23.77	15q23
19	AGTRL1 **1** (32)	angiotensin II receptor-like 1	22.36	11q12
22	CD44	Homo sapiens CD44 isoform RC (CD44) mRNA, complete cds	19.88	11p13
24	MYT1	myelin transcription factor 1	18.49	20q13.33
26	TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)	17.53	3q23-q24
31	TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	16.23	Xp11.3-p11.23
32	HLA-DPA1 **3** (1, 2, 32)	major histocompatibility complex, class II, DP alpha 1	15.79	6p21.3
36	S100A10	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	15.27	1q21
38	TLR2 **1** (14)	toll-like receptor 2	14.66	4q32
40	TFPI **1** (12)	ESTs, Weakly similar to cytokine receptor-like factor 2; cytokine receptor CRL2 precusor [Homo sapiens] [H.sapiens]	14.09	2q32
44	POSTN	osteoblast specific factor 2 (fasciclin I-like)	12.60	13q13.1
46	CHI3L2	chitinase 3-like 2	12.22	1p13.3
48	CCL3	chemokine (C-C motif) ligand 3	11.89	17q11-q21
49	MSR1	macrophage scavenger receptor 1	11.87	8p22
50	SRPX	sushi-repeat-containing protein, X chromosome	11.29	Xp21.1
51	CSPG2 **1** (2)	chondroitin sulfate proteoglycan 2 (versican)	10.86	5q14.3
56	CD151	CD151 antigen	10.61	11p15.5
-74	FGF13 **2** (5, 6)	fibroblast growth factor 13	-10.34	Xq26.3
-72	MEF2C **1** (5)	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	-10.48	5q14
-70	WNT10B **2** (28,46)	wingless-type MMTV integration site family, member 10B	-10.50	12q13
-69	NPY1R **1** (32)	neuropeptide Y receptor Y1	-10.96	4q31.3-q32

-67	ATP2B2 **1** (19)	Homo sapiens, Similar to nuclear localization signals binding protein 1, clone IMAGE:4933343, mRNA	-11.22	3p25.3
-66	PRKCB1 **15** (3-5, 7, 8, 10, 16-19, 22, 28, 36,39,41)	protein kinase C, beta 1	-11.24	16p11.2
-65	MAL	mal, T-cell differentiation protein	-11.55	2cen-q13
-62	CACNA2D3 **1** (5)	calcium channel, voltage-dependent, alpha 2/delta 3 subunit	-11.89	3p21.1
-60	GRIN1 **3** (7, 19, 32)	glutamate receptor, ionotropic, N-methyl D-aspartate 1	-12.15	9q34.3
-59	HTR2A **3** (10,19, 32)	5-hydroxytryptamine (serotonin) receptor 2A	-12.22	13q14-q21
-55	SNAP25 **1** (43)	synaptosomal-associated protein, 25kDa	-13.04	20p12-p11.2
-54	PTK2B **4** (8, 16, 17, 19)	PTK2B protein tyrosine kinase 2 beta	-13.42	8p21.1
-52	VIP **1** (32)	vasoactive intestinal peptide	-14.27	6q26-q27
-50	VAMP1 **1** (43)	vesicle-associated membrane protein 1 (synaptobrevin 1)	-15.17	12p
-48	NPY **2** (32, 35)	neuropeptide Y	-15.63	7p15.1
-45	ENPP2	ectonucleotide pyrophosphatase/ phosphodiesterase 2 (autotaxin)	-17.09	8q24.1
-44	MBP	myelin basic protein	-17.48	18q23
-39	HTR2C **3** (10, 1, 32)	5-hydroxytryptamine (serotonin) receptor 2C	-19.23	Xq24
-37	P2RX5 **2** (19,23)	purinergic receptor P2X, ligand-gated ion channel, 5	-19.57	17p13
-32	MATK **2** (6, 15)	megakaryocyte-associated tyrosine kinase	-23.75	19p13.3
-31	CRH **2** (22, 32)	corticotropin releasing hormone	-24.27	8q13
-22	NEFH **2** (25,31)	neurofilament, heavy polypeptide 200kDa	-27.40	22q12.2
-21	ICAM5	intercellular adhesion molecule 5, telencephalin	-27.70	19p13.2
-19	EGR4	early growth response 4	-28.90	2p13
-15	CAMK2A **5** (7, 8, 19, 20, 28)	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	-36.76	5q33.1
-13	ATP2B2 **1** (19)	ATPase, Ca++ transporting, plasma membrane 2	-39.22	3p26-p25
-11	SST **1** (32)	somatostatin	-43.86	3q28
-10	ITPKA **2** (3, 19)	inositol 1,4,5-trisphosphate 3-kinase A	-49.50	15q14-q21
-6	GABRA5 **1** (32)	gamma-aminobutyric acid (GABA) A receptor, alpha 5	-101.01	15q11.2-q12
-3	CACNG3 **1** (5)	calcium channel, voltage-dependent, gamma subunit 3	-136.99	16p12-p13.1
-2	CCK **1** (32)	cholecystokinin	-146.63	3p22-p21.3
-1	FOXG1B	forkhead box G1B	-325.73	14q12-q13

The numbers outside the brackets after the gene symbol, indicate how many specific pathways the gene is known to be involved in. The numbers inside the brackets correspond to the specific pathways in Table 4.3. Genes highlighted in red are involved in many pathways shown by Table 4.4.

Table 4.3 The Onto-tools result ranking KEGG pathways disrupted by the 1844 differentially expressed genes in pilocytic astrocytoma.

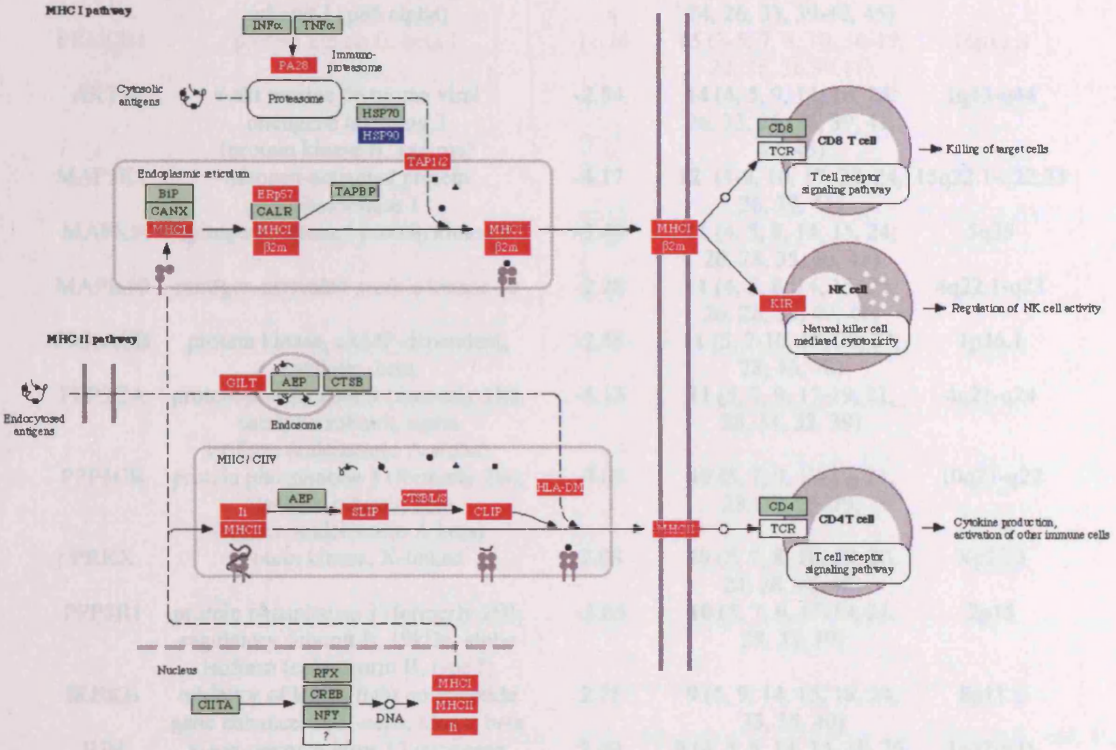
Rank	Unique Pathway-id	KEGG Database Pathway Name	Impact Factor	Genes in Pathway	Pathway Genes in Input (percentage %)
1	1:04612	Antigen processing and presentation	76.6	86.0	24.4
2	1:04514	Cell adhesion molecules (CAMs)	24.1	132.0	25.8
3	1:04070	Phosphatidylinositol signalling system	23.2	79.0	27.8
4	1:04510	Focal adhesion	14.7	194.0	25.8
5	1:04010	MAPK signalling pathway	14.3	273.0	22.7
6	1:04810	Regulation of actin cytoskeleton	12.9	206.0	23.3
9	1:04210	Apoptosis	9.3	84.0	27.4
10	1:04540	Gap junction	9.3	99.0	25.3
11	1:04512	ECM-receptor interaction	9.1	87.0	26.4
12	1:04610	Complement and coagulation cascades	8.8	69.0	27.5
14	1:04620	Toll-like receptor signalling pathway	8.1	91.0	25.3
16	1:04670	Leukocyte transendothelial migration	7.8	117.0	23.1
17	1:04650	Natural killer cell mediated cytotoxicity	7.3	128.0	21.1
18	1:04662	B cell receptor signalling pathway	7.2	63.0	27.0
19	1:04020	Calcium signalling pathway	7.0	176.0	18.2
24	1:04910	Insulin signalling pathway	5.7	135.0	20.7
25	1:01510	Neurodegenerative disorders	5.7	35.0	28.6
26	1:05210	Colorectal cancer	5.6	77.0	23.4
28	1:04310	Wnt signalling pathway	5.5	147.0	18.4
32	1:04080	Neuroactive ligand-receptor interaction	4.7	302.0	11.3
33	1:04660	T cell receptor signalling pathway	4.4	93.0	20.4
35	1:04920	Adipocytokine signalling pathway	4.0	69.0	20.3
36	1:04530	Tight junction	3.9	119.0	16.8
37	1:04520	Adherens junction	3.8	77.0	20.8
38	1:04110	Cell cycle	3.5	112.0	16.1
39	1:04370	VEGF signalling pathway	3.0	72.0	16.7
42	1:04150	mTOR signalling pathway	2.6	49.0	18.4
43	1:04130	SNARE interactions in vesicular transport	2.5	36.0	13.9
44	1:04350	TGF-beta signalling pathway	2.3	84.0	15.5
45	1:04630	Jak-STAT signalling pathway	2.2	153.0	13.7
46	1:04340	Hedgehog signalling pathway	1.6	57.0	12.3
47	1:04060	Cytokine-cytokine receptor interaction	1.6	256.0	11.7
51	1:04140	Regulation of autophagy	0.8	29.0	3.4
52	1:04330	Notch signalling pathway	0.8	46.0	8.7

The pathways are ranked according to impact factor. This impact factor takes into account the fold change and number of genes differentially expresses in a pathway and the influence each individually disrupted gene has on a pathway.

The pathway most affected by differential gene expression (rank 1) was antigen processing and presentation, in which the majority of genes are significantly up-regulated and located at 6p21.3 (Figure 4.3). Pathways expected to have a greater involvement in tumour development than indicated by their rank appear to have a lower percentage of pathway input genes. These include the calcium signalling pathway, WNT signalling pathway, the cell cycle, TGF-beta signalling pathway, Jak-STAT

signalling pathway and the Notch signalling pathway that all have a gene input less than 20%.

Figure 4.3 The KEGG pathway antigen processing and presentation ranked first by the Onto-tools software.



This pathway illustrates the aberrantly expressed genes in paediatric PAI involved in antigen processing and presentation. Those genes showing up-regulation are red and those down-regulated are blue.

To investigate if particular differentially expressed genes could influence several pathways and have an overall impact on tumour development, those genes involved in 5 or more pathways were identified (Table 4.4). Although *MAPK1* is involved in 17 pathways, this gene only showed a 2.01-fold down-regulation in expression. In contrast, genes with the largest fold changes are involved in fewer pathways. Only two genes, *CAMK2A* and *PRKCB1,* showed a greater than 10-fold change in expression and are involved in more than 5 pathways (Tables 4.2 and 4.4), suggesting that those genes with the highest degree of deregulation in paediatric PAI may not have the greatest impact on cellular pathways.

Table 4.4 The Onto-tools result of differentially expressed genes involved in 5 pathways or more.

Gene symbol	Gene description	Fold change in expression	Number of pathways gene involved in	Chromosome location
MAPK1	mitogen-activated protein kinase 1	-2.01	**17** (4-8, 10,17,21,22, 24, 26, 37, 39-42, 44)	22q11.21
PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide	-2.05	**16** (3, 4, 6, 9, 14, 16-18, 24, 26, 33, 39-42, 45)	3q22.3
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	-2.13	**16** (3, 4, 6, 9, 14, 16-18, 24, 26, 33, 39-42, 45)	5q13.1
PRKCB1	protein kinase C, beta 1	-11.24	**15** (3-5, 7, 8, 10, 16-19, 22, 28, 36,39,41)	16p11.2
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	-2.54	**14** (4, 5, 9, 14, 18, 24, 26, 33, 35, 36, 39, 41, 42, 45)	1q43-q44
MAP2K1	mitogen-activated protein kinase kinase 1	-4.17	**12** (4-8, 10, 17, 22, 24, 26, 39, 41)	15q22.1-q22.33
MAPK9	mitogen-activated protein kinase 9	-3.48	**11** (4, 5, 8, 14, 15, 24, 26, 28, 35, 40, 41)	5q35
MAPK10	mitogen-activated protein kinase 10	-2.28	**11** (4, 5, 8, 14, 15, 24, 26, 28, 35, 40, 41)	4q22.1-q23
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	-2.55	**11** (5, 7-10, 19, 20, 24, 28, 46, 48)	1p36.1
PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	-4.13	**11** (5, 7, 9, 17-19, 21, 28, 31, 33, 39)	4q21-q24
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)	-3.65	**10** (5, 7, 9, 17-19, 21, 28, 31, 33, 39)	10q21-q22
PRKX	protein kinase, X-linked	2.08	**10** (5, 7, 8, 10, 19, 20, 24, 28, 46, 48)	Xp22.3
PPP3R1	protein phosphatise 3 (formerly 2B), regulatory subunit B, 19kDa, alpha isoform (calcineurin B, type I)	-3.05	**10** (5, 7, 9, 17-19, 21, 28, 33, 39)	2p15
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	2.75	**9** (5, 9, 14, 15, 18, 24, 33, 35, 40)	8p11.2
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	2.49	**9** (4, 5, 8, 14, 15, 18, 26, 28, 33)	1p32-p31
PAK1	*CDKN1A* /Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	-7.87	**7** (4-6, 15, 17, 21, 33)	11q13-q14
TP53	tumor protein p53 (Li-Fraumeni syndrome)	3.14	**7** (5, 9, 26, 28, 29, 31, 38)	17p13.1
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-2.73	**7** (3, 7, 8, ,10, 19, 22, 28)	20p12
CALM1	calmodulin 1 (phosphorylase kinase, delta)	-2.22	**7** (3, 7, 8, 19, 20, 24, 29)	14q24-q31
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	5.76	**7** (4-6, 10, 19, 26, 47)	4q11-q13
CALM3	calmodulin 3 (phosphorylase kinase, delta)	-2.31	**7** (3, 7, 8, 19, 20, 24, 29)	19q13.2-q13.3
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2.26	**6** (2, 4, 6, 11, 16, 21)	10p11.2
ITPR1	inositol 1,4,5-triphosphate receptor, type 1	-8.62	**6** (3, 7, 8, 10, 19, 22)	3p26-p25
ITPR2	inositol 1,4,5-triphosphate receptor, type 2	5.42	**6** (3, 7, 8, 10, 19, 22)	12p11
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	2.42	**6** (4, 6, 16, 21, 28, 44)	18q11.1
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.17	**6** (9, 14, 15, 18, 33, 35)	14q13
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	-2.40	**6** (4, 6, 16, 21, 28, 44)	2p24

CCND1	cyclin D1	3.77	**5** (4, 26, 28, 38,45)	11q13
CAMK2A	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	-36.77	**5** (7, 8, 19, 20, 28)	5q33.1
CAMK2B	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	-8.77	**5** (7, 8, 19, 20, 28)	7p14.3-p14.1
GNAI3	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	2.72	**5** (10, 16, 21, 22, 36)	3p21
TGFB1	transforming growth factor, beta 1(Camurati-Engelmann disease)	2.32	**5** (5, 26, 38, 44, 47)	19q13.1
ACTN1	actinin, alpha 1	2.26	**5** (4, 6, 16, 36, 37)	14q22-q24
PAK2	CDKN1A-activated kinase 2	2.23	**5** (4-6, 21, 33)	3q29
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	5.10	**5** (5, 14, 18, 26, 33)	14q24.3
ACTN2	actinin, alpha 2	-2.52	**5** (4, 6, 16, 36, 37)	1q42-q43
MAP2K4	mitogen-activated protein kinase kinase 4	-3.30	**5** (5, 8, 14, 15, 41)	17p11.2
PLA2G6	phospholipase A2, group VI (cytosolic, calcium-independent)	-2.02	**5** (5, 8, 2, 39, 41)	22q13.1
PTPN6	protein tyrosine phosphatase, non-receptor type 6	2.21	**5** (17, 18, 33, 37, 45)	12p13
FAS	Fas (TNF receptor superfamily, member 6)	5.90	**5** (5, 9, 13, 17, 47)	10q24.1
CCND2	cyclin D2	2.02	**5** (4, 26, 28, 38, 45)	12p13
PLA2G12A	phospholipase A2, group XIIA	3.99	**5** (5, 8, 22, 39, 41)	4q25
GNAS	GNAS complex locus	-4.93	**5** (8, 10, 19, 22, 48)	20q13.3
GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	-2.25	**5** (10, 16, 21, 22, 36)	7q21
CAMK2G	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	-3.40	**5** (7, 8, 19, 20, 28)	10q22

The numbers in bold outside the brackets correspond to the specific pathways in Table 4.3 and the numbers inside the brackets are the pathway rankings. Genes highlighted in red are the most disrupted in PAI, with a greater than 10-fold change in expression. These genes are listed in Table 4.2.

Validation of array results using Q-PCR

To validate the expression results obtained from the Affymetrix GeneChip® experiments, the expression of 6 genes that showed a 2-fold and significant difference in expression in PAI compared to the normal controls were assessed using Q-PCR in 16 of the 19 samples. Sufficient RNA was not available for the remaining 3 samples. The array data demonstrated that *CCNA1, SPINT2, MAPK1* and *MATK* were significantly down-regulated and *TIMP1* and *MCL1* were significantly up-regulated. Q-PCR confirmed these changes in gene expression in PAI compared to normal brain (Table 4.5). Similar differential expression of these genes was also found when SAGE data was analysed (http:cgap.nci.nih.gov) (Boon et al., 2002b). Only data from paediatric astrocytoma tumours were included.

Table 4.5 Q-PCR validation of the microarray data.

Tumour Sample	TIMP1	MCL1	SPINT2	CCN1A	MAPK1	MATK
	Array/Q-PCR	Array/Q-PCR	Array/Q-PCR	Array/Q-PCR	Array/Q-PCR	Array/Q-PCR
IN1350	10.95/5.46	2.88/1.89	0.24/0.02	0.19/0.00	0.50/0.16	0.04/0.01
IN1740	43.15/35.92	2.281.89	0.18/0.03	0.28/0.00	0.57/0.26	0.03/0.01
IN2110	12.11/9.60	2.33/1.39	0.31/0.03	0.03/0.00	0.43/0.20	0.03/0.01
IN2368	10.20/20.49	5.63/2.34	0.07/0.02	0.17/0.00	0.67/0.29	0.05/0.01
IN2524	13.52/9.54	6.42/1.53	0.24/0.03	0.17/0.00	0.68/0.26	0.03/0.00
IN2604	26.74/20.16	5.25/2.44	0.17/0.02	0.19/0.02	0.85/0.11	0.07/0.00
IN2631	18.05/5.58	6.15/0.78	0.25/0.02	0.32/0.01	0.72/0.17	0.02/0.00
IN2674	7.12/6.26	3.43/1.50	0.57/0.16	0.25/0.02	0.50/0.46	0.03/0.03
IN2825	27.41/32.00	5.43/3.19	0.17/0.03	0.40/0.05	0.46/0.22	0.06/0.01
IN2921	39.89/26.05	3.68/1.16	0.60/0.05	0.14/0.00	0.37/0.13	0.05/0.01
IN2940	17.56/7.78	4.93/1.30	0.28/0.04	0.22/0.00	0.38/0.17	0.03/0.01
IN2969	11.15/9.89	3.57/1.68	0.26/0.05	0.26/0.03	0.48/0.27	0.04/0.01
IN3013	14.69/13.42	3.95/1.33	0.33/0.05	0.14/0.00	0.49/0.17	0.05/0.00
IN3017	13.95/23.92	3.15/3.50	0.13/0.05	0.22/0.01	0.60/0.32	0.04/0.00
IN3126	11.68/11.69	7.56/1.00	0.38/0.08	0.31/0.00	0.67/0.26	0.05/0.01
IN3156	30.88/19.93	3.91/0.89	0.30/0.04	0.26/0.03	0.45/0.18	0.02/0.01

The comparative C_T method was used to determine the relative ratio of expression results for each gene using Q-PCR. In this approach β-actin was used as the endogenous control and adult normal total brain was used as the control. The relative quantitative value shown above is expressed as $2^{-\Delta\Delta C_T}$.

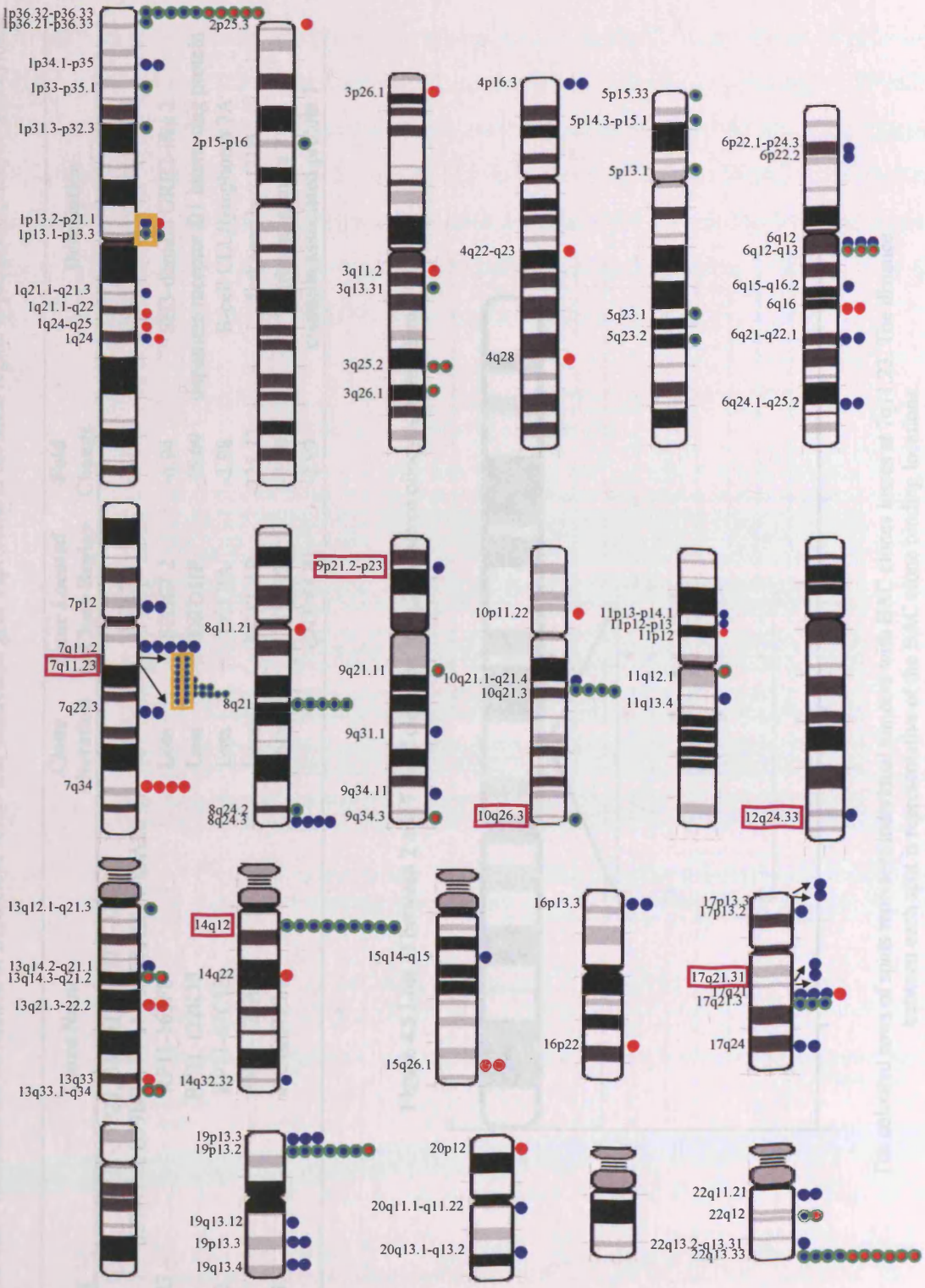
Genomic aberrations found in paediatric pilocytic astrocytoma biopsies

aCGH was carried out using the SpectralChip™ 2600 array to identify regions of chromosome gain and loss in 11 paediatric PAI. In total, 97 individual BAC clones were found to be either gained or lost in 1 or more tumours. Of these, 26 were gained, 64 were lost and 7 were gained or lost in different tumours (Figure 4.4) (The raw data for this analysis can be found on the Supplementary Data CD included with this thesis). Several chromosome regions were frequently altered in PAI. BAC clones mapping to 1p36.33, 14q12, 19p13.2 and 22q13.33 showed either gain, loss or both in 91%, 82%, 64% and 91% of tumours, respectively. A small region containing 2 adjacent clones at 1p13 was lost in 1 tumour and a region that varied in size containing up to 7 adjacent clones at 7q11.23 was lost in 7 tumours (Figure 4.5). Other regions less frequently altered included 7q11.2 and 8q21 in 45% of tumours, 7q34, 8q24.3, 10q21.3 and 17q21 in 36% of tumours and 6q12, 6q13, 17q21.3 and 19p13.3 in 27% of tumours.

Reliable gene expression data was not available for all genes located in the same regions as the 97 altered clones. Overall, loss of six individual clones correlated with down-regulation of genes mapping to the same region, comprising *BCL7B* at 7q11.23 in 7 tumours (Figure 4.5); *SH3GL2* at 9p21.2-p23 in 1 tumour; *BCL7A* at 12q24.33 in 1 tumour; *DRD1IP* at 10q26.3 in 1 tumour; and *TUBG2* and *CNTNAP1* at 17q21.31 in 2 tumours. Critically, although clone loss at these regions was not seen in every tumour, all tumours showed a 2-fold and significant down-regulation in the specific genes at these loci (Table 4.6) No correlations were found between clone gain and gene over-expression.

Large scale copy number variations (LCVs) in specific chromosome regions have been characterised in the normal population. Of the 97 clones that were involved in CNAs, 37 are located in regions of known LCV, indicated by the green circles in Figure 4.4.

Figure 4.4 aCGH results indicating regions of chromosome gain and loss in paediatric PAI.

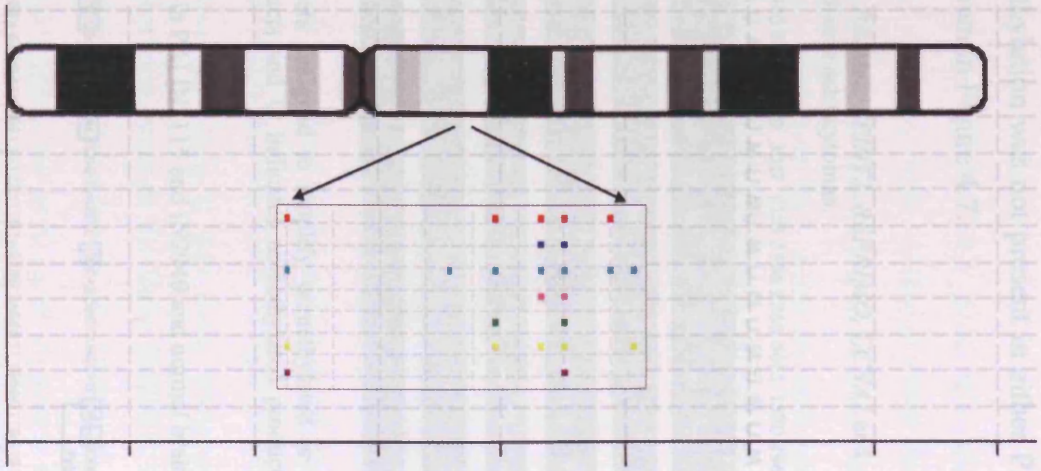


Circles to the right of each chromosome ideogram show the number of individual tumours with single clone gains (red) and losses (blue) among the 11 PAI studied. Clones in a region of LCV in the normal population are indicated by a green ring. Adjacent clones altered in the same tumour are indicated by a yellow box. Multiple clones in a region are indicated by two arrows. Cytogenetic band positions are shown to the left. Correlations between clone alterations and differential gene expression in the same region are indicated by a purple box.

Table 4.6 The Table illustrates the correlation of BAC clone losses and differential gene expression in the same region in paediatric pilocytic astrocytoma.

Clone Location	Clone Name	Clone Alteration	Gene Located in Clone Region	Fold Change	Description
7q11.23	RP11-35P20, B315H11, CITB-51J22, B270D13, B39H04, RP11-137E8, RP11-89A20	Loss	BCL7B	-2.14	B-cell CLL/lymphoma 7B
9p21.2-p23	RP11-163F8	Loss	SH3GL2	-6.94	SH3-domain GRB2-like 2
10q26.3	RP11-122K13	Loss	DRD1IP	-35.09	dopamine receptor D1 interacting protein
12q24.33	RP11-87C12	Loss	BCL7A	-2.98	B-cell CLL/lymphoma 7A
14q12	RP11-125A5	Loss	FOXG1B	-325.73	forkhead box G1B
17q21.31	AC100793.8	Loss	TUBG2	-2.28	tubulin, gamma 2
			CNTNAP1	-2.99	contactin associated protein 1

Figure 4.5 Loss of between 2 and 7 BAC clones at 7q11.23 in seven pilocytic astrocytoma.

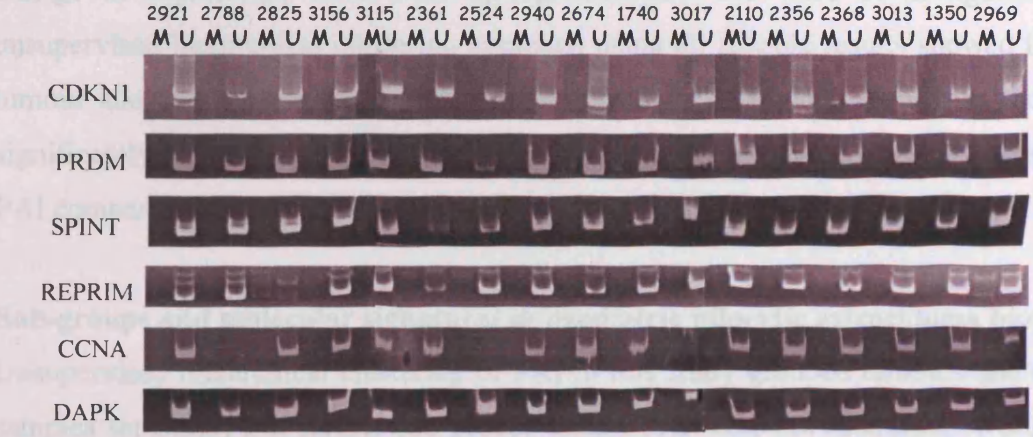


The coloured rows of spots represent individual tumours with BAC clones losses at 7q11.23. The distance between each spot is representative of the BAC clone binding locations.

Methylation specific PCR and sequencing in paediatric pilocytic astrocytoma biopsies

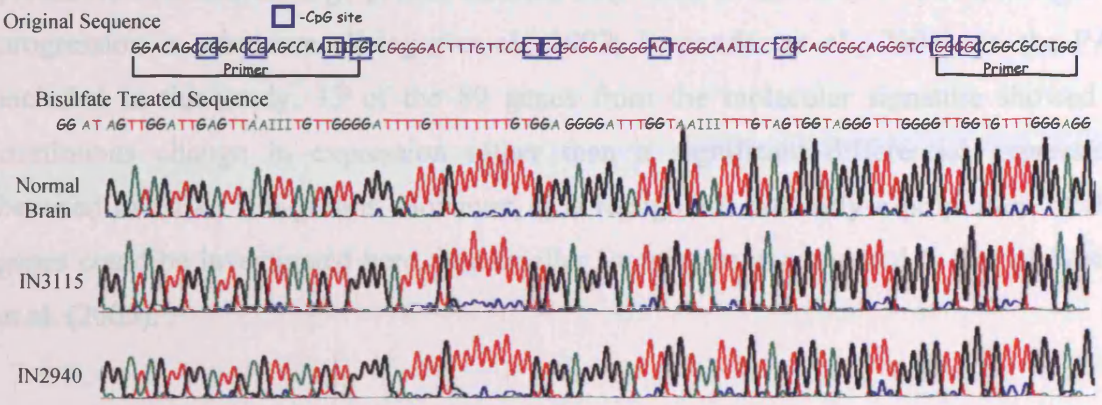
MSP products were only generated by primer sets specific for unmethylated promoter regions, indicating that the promoter regions of *CDKN1C, PRDM2, SPINT2, REPRIMO, CCNA1* and *DAPK1* were not methylated in the 17 PAI analysed (Figure 4.6). A result was not achieved for *CCNA1* in tumours IN2788, IN2825 and IN3017 despite repeated attempts. Sequencing of each gene in IN3115 and IN2940 and normal brain confirmed that promoter region methylation was not present in either PAI or the normal samples. An example of this is shown in Figure 4.7.

Figure 4.6 MSP results for *CDKN1C, PRDM2, SPINT2, REPRIMO, CCNA1* and *DAPK1* in paediatric astrocytomas.



These results show PCR products for primer sets designed to amplify unmethylated or methylated promoter regions. M indicates methylated products and U indicates unmethylated products for each tumour.

Figure 4.7 Sequencing data for DAPK1 in PAI IN3115 and IN2940 and normal brain.



CpG sites where methylation could occur are indicated in the original sequence by a blue box. Unmethylated cytosine residues are converted to uracil residues which during amplification become thymidine residues. The bisulfite treated sequence illustrates no methylation.

DISCUSSION

This Chapter has focused on global differential gene expression in PAI and correlations between genomic CNAs at a 1MB resolution in order to identify and characterise cellular pathways that are involved in tumour development and growth. Genomic gain and loss and gene promoter region methylation are mechanisms that disrupt gene expression and play a significant role in the development of many tumours.

Using the Affymetrix GeneChip® U133A array, 10563 reliable gene expression results were generated for 19 paediatric PAI and 4 normal brain controls. From these, 1844 differentially expressed genes were identified that showed a 2-fold and significant change in expression; 1002 were up-regulated and 842 were down-regulated. An unsupervised hierarchical clustering approach using all reliable results showed that the tumour and control samples grouped separately. No GO biological process showed a significantly large difference in the percentage of differentially expressed genes in the PAI compared to normal controls.

Sub-groups and molecular signatures in paediatric pilocytic astrocytoma biopsies

Unsupervised hierarchical clustering of PAI in this study grouped tumours and control samples separately but further sub-groups within PAI were not identified. Wong et al. (2005) previously demonstrated that two sub-groups of PAI could be distinguished by a molecular signature of specific genes and suggested that these sub-groups represented PAI with different potentials of progression. While it has been documented that paediatric PAI that undergo partial excision may recur in the same location, malignant progression is very rare (Krieger et al., 1997; Fernandez et al., 2003). In the PAI included in this study, 13 of the 89 genes from the molecular signature showed a continuous change in expression rather than a significant differential expression between two PAI sub-groups. However, it is recognised that only a proportion of the genes could be investigated here, in a smaller sample group compared to that of Wong et al. (2005).

Sharma et al. (2007) also reported a molecular signature of 36 genes that showed differential expression between supratentorial PAI and posterior fossa PAI and

suggested that glial tumours have an intrinsic, lineage specific signature, which reflects the region of the brain from which the non-malignant tumour cell precursors originate. In the present tumour group, the majority of tumours were located in the posteria fossa, consequently a comparison to this study was not possible. These studies emphasises the intrinsic genetic heterogeneity of PAI and the need for large sample numbers to identify true sub-groups with clinical or prognostic relevance.

Genes involved in paediatric pilocytic astrocytoma development

To investigate paediatric PAI development, those genes which showed a greater than 10-fold change in expression and were either involved in a KEGG pathway or are well-characterised were identified. The most over-expressed gene, *SERPINA3,* is specifically expressed in astrocytes and is involved in the expression of astrocytic markers such as GFAP (Gopalan et al., 2006a; Gopalan et al., 2006b). In this study, *GFAP* was 1.98-fold up-regulated just missing the 2-fold cut-off for further analysis. These results support that the tumour cells are of astrocytic lineage. The second most over-expressed gene *CHI3L1 (*also known as *YKL-40)* is another astrocytic marker and has previously been used as a diagnostic marker in astrocytoma (Nutt et al., 2005; Rousseau et al., 2006).

CHAD and *TIMP4* have been shown to be over-expressed in PAI compared to other grades of astrocytoma and normal brain in two other studies (Rickman et al., 2001; Rorive et al., 2006). Interestingly, the proteins of these genes inhibit migration and may contribute to the benign phenotype of PAI. Rickman et al. (2001) also found over-expression of *CSPG4, CPGS2* and *FOS* and under-expression of *PRKACB* as found in the PAI of this study (Tables 4.2 and 4.4).

Several genes from Table 4.2 have already been investigated in other grades of astrocytoma. These include *T1A-2, ABCC3, CD44, MYT1, TIMP1, HLA-DPA1, TFPI, CSPG2, CD151, WNT10B, PTK2B, ENPP2, MBP, MATK, SST, CCK* and *FOXG1B.* The expression of *T1A-2* (also known as *podoplanin*) was found to be significantly higher in GBMIV compared to AAIII or DAII and is thought to be a marker of malignant progression (Mishima et al., 2006). The large fold change seen here compared to normal brain suggests that this gene is involved in promoting tumour development in PAI. *ENPP2* also known as *autotaxin* is highly expressed in GBMIV and involved in the promotion of cell motility (Kishi et al., 2006). This gene is

significantly down-regulated in the PAI of this study possibly hindering cell motility. Other genes from this group have functions associated with a range of tumour characteristics. For example, increased expression of *ABCC3,* from the multi-drug resistance protein family of ATP-dependent efflux pumps, may mediate drug resistance in PAI (Bronger et al., 2005). The up-regulation of TIMP1 and HLA-DPA1, immune defence-associated genes, may contribute to the benign biological behaviour of this tumour (Huang et al., 2005). There is evidence to suggest that *CSPG2* (also known as *versican*) protects from oxidative-stress induced apoptosis and promotes tumour growth and angiogenesis (Zheng et al., 2004). Furthermore, *CPGS4* from the same family was found to be up-regulated in diffuse and malignant astrocytoma in response to PDGF and increases tumour cell proliferation and invasion (Chekenya et al., 1999; Johansson et al., 2005). In the present study, *CSPG2* and *CPGS4* demonstrated a 10.86 and 23.77-fold up-regulation in expression respectively, in addition to *PDGFRα* which was also over-expressed by 5.84-fold.

The additional genes in Table 4.2 are either associated with the development of other tumours and cancers or have functions that could promote or inhibit tumour development. *POSTN* is over-expressed in more than 80% of human colon cancers and demonstrates the highest fold change expression in oral squamous-cell carcinoma (OSCC). *POSTN* enhances cancer growth by both preventing stress-induced apoptosis and augments endothelial cell survival to promote angiogenesis (Bao et al., 2004; Siriwardena et al., 2006). Furthermore, the homophilic interactions of *CD151* have been shown to stimulate integrin-dependent signalling to c-Jun, enhancing cell motility in melanoma (Hong et al., 2006). *CD151* is up-regulated in PAI and could increase cell motility through a similar mechanism. *ITPKA* is significantly down-regulated in the PAI studied here and OSCC cell lines. It is involved in the phosphorylation of inositol 1,4,5-trisphosphate that regulates the intracellular calcium (Ca2+) concentrations by releasing Ca2+ from intracellular stores. *ITPKA* is also responsible for regulating the levels of a large number of inositol polyphosphates that are involved in cellular signalling. Disruption of this gene and consequently intracellular signalling could be linked to PAI development (Kato et al., 2006).

The study by Wong et al. (2005) also identified 428 genes that showed a significant and 3-fold change in expression from the normal, 192 up-regulated and 236 down-regulated.

Of the 428 genes that showed differential expression, 96 were sub-grouped according to biological processes. A total of 23 genes were involved in neurogenesis, 7 of which have been identified in this study as showing a 2-fold and significant change in expression; *DLG4, GAS7, SEMA3E, HEY2, ASCL1, DPYSL3* and *SEMA5A.* A further 20 genes were involved in cell adhesion and 3 were also identified in this study; *COL9A1*, *ITGB2* and *SEMA5A.* 14 genes involved in synaptic transmission were identified and 4 corresponded with this study; *GLG4, GRM3, SYT1* and *GABRAG2.* A group of 11 genes were involved in CNS development and only 2 were also identified in this study; *DCAMKL1* and *SH3GL3.* 10 genes were involved in potassium ion transport and only 1 corresponded with this study; *ATP1B1.* Genes involved in protein amino acid dephosphorylation formed a group of 10 and 2 were also identified in this study; *DUSP8* and *PPP3CA.* Finally 8 genes were involved in cell differentiation and only 1 was also identified in this study; *ASCL1.*

Pathways with differential gene expression in paediatric pilocytic astrocytoma biopsies

The Onto-tool software ranked the KEGG pathways influenced by the 1844 differentially expressed genes according to the extent of disruption. In PAI, the KEGG pathway antigen processing and presentation was most affected by differential gene expression. With the exception of *HSP90A,* all identified genes involved in this pathway were up-regulated, including *CD74, HLA-DPA1, HLA-DRA, HLA-DMA, HLA-DRB1, HLA-DMB, HLA-DQB1, HLA-G, CTSS, HLA-B, KLRC3, HLA-F, HLA-A, PSME2, PDIA3, HLA-E, IFI30, TAP1*, and *B2M.* A previous study observed an increase in the expression of genes involved in immune response in PAI compared to DAII, oligodendrogliomas and normal brain (Huang et al., 2005). An increase in *HLA-DPA1* expression in PAI compared to AAIII was also observed in a second study (Hunter et al., 2002).

The major histocompatibility complex molecules also known as the HLA system have a crucial role in antigen processing and presentation regulating T lymphocytes and influencing immunosurveillance. The six HLA-D molecules are known as MHC class II and are classically expressed on specialised antigen presenting cells such as macrophages and dendritic cells. These cells present antigen to T (CD4+) lymphocytes activating an immune response. The increase of MHC class II expression in

premalignant lesions of breast, colon, cervix and larynx appears to be independent of leukocytic infiltration (Garrido et al., 1993). This has also been previously documented in low-grade astrocytoma (Rossi et al., 1988). The biopsy samples of this study were directly adjacent to tumour tissue processed for routine histological evaluation, which were first examined macroscopically to ensure that no frankly normal tissue was present. However, increased numbers of immune cells may have been present in the tissue used. Moreover, astrocytes have also been shown to express class II major histocompatibility complex (MHC) antigens and costimulatory molecules (B7 and CD40) that are critical for antigen presentation and T-cell activation, suggesting that the up-regulation of the antigen processing and presentation pathway in these PAI maybe a response of the astrocytoma cells (Dong and Benveniste, 2001). Furthermore, HLA-DR over-expression has been associated with good prognosis in large bowel carcinoma and ovarian tumours (Lovig et al., 2002; Tamiolakis et al., 2003). The up-regulation of immune response genes in PAI may contribute to the benign behaviour of this tumour type.

The KEGG pathways phosphatidylinositol signalling (PI3K) and MAPK signalling were ranked as third and fifth respectively, according to the extent of differential gene expression in PAI (Table 4.3). Altered signalling in these pathways through increased receptor stimulation and disrupted tumour suppressor or oncogene function has been characterised in other grades of astrocytoma particularly primary and secondary adult GBMIV (Soni et al., 2005).

Of the 22 genes differentially expressed in the PI3K pathway, 19 (86%) are down-regulated. Similarly in the MAPK signalling pathway, 58% (36/62) of genes are under-expressed compared to normal brain. Of those genes involved in 10 or more pathways, 85% (11/13) are involved in the MAPK signalling pathway and 91% (10/11) of these are down-regulated including *MAPK1*, *PRKCB1*, *AKT3*, *MAP2K1*, *MAPK9* and *MAPK10* (Table 4.4). Moreover, *EGFR* commonly over-expressed or amplified in adult primary GBMIV, showed a 5.21 down-regulation in expression. In contrast, several growth factors and corresponding receptors involved in the propagation and stimulus of the PI3K or MAPK signalling pathways are over-expressed, including *PDGFRA*, *ITGB1*, *TGFB1*, *FAS*, *PDGFC*, *FGFR1*,*FGF2*, *TNF1A* and *IL1R1*. Furthermore, three key target transcription factors *JUN*, *FOS* and *TP53* activated by the MAPK signalling

pathway are also up-regulated. AP-1 dimers are a group of proteins involved in the control of cell proliferation, neoplastic transformation and apoptosis. Jun and Fos are a group of transcription factors capable of forming the dimers of AP-1. Most evidence suggests that cell fate is influenced by AP-1 through the regulation of cell cycle components (Shaulian and Karin, 2001a). AP-1 is known to directly induce the transcription of *cyclin D1,* a gene found to be up-regulated 3.77-fold in this study. The growth factor and receptor up-regulation in the PI3K and MAPK signalling pathways is contradictory to the reduction of gene expression and overall pathway activity, suggesting that the up-regulation of target transcription factors may occur through a different mechanism or pathway.

Genomic loss in regions of large scale copy number variations in paediatric pilocytic astrocytoma biopsies

Of the 97 BAC clones found to be altered in PAI, 40% were located in regions of LCV present in the normal population (Iafrate et al., 2004; Sebat et al., 2004; Sharp et al., 2005; Locke et al., 2006; Fiegler et al., 2006; Khaja et al., 2006; Redon et al., 2006; Conrad et al., 2006). The role of LCVs (which involve gain or loss of hundreds of kilobases of genomic DNA) in phenotypically normal individuals is not clear (Iafrate et al., 2004). However, LCV at 14q12 was present in DNA from 50% of chronic myeloid leukaemia (CML) and paediatric solid tumours compared to an incidence of 10% in somatic DNA of healthy control individuals, suggesting that acquired or inherited LCVs at 14q12 may be associated with the onset or progression of neoplasia (Braude et al., 2006). In this study, the incidence of 14q12 loss in PAI was 82%. Further investigations are necessary to establish the incidence of LCVs in somatic DNA of PAI patients in order to determine whether 14q12 alterations are inherited or are acquired during tumour development.

It would be particularly valuable if the alterations located in regions of LCV could be characterised as either being present in somatic DNA or only found in tumour DNA. Additional aCGH experiments investigating the normal blood from the patients of these tumours 19 PAI could determine whether these alterations were inherited or acquired. Normal blood is available from these patients; however limited funding prevented further investigation of these regions.

Correlation of copy number alterations and differential gene expression in paediatric pilocytic astrocytoma biopsies

Three genes are located in the exact region of loss at 14q12, including *RPL26P*, *BTF3P3* and *LOC387978*. *FOXG1B* is adjacent to the clone at 14q12 and is the only gene with reliable gene expression data in this study. *FOXG1B* expression was significantly down-regulated in all PAI compared to normal controls ($p = 0.01$).

FOXG1B, also known as *BF1*, *FKH2*, *FKHL1*, *FKHL4*, *HBF-1*, *HFK1* or *QIN*, has many functions. In the embryo, *FOXG1B* expression is localised in progenitor cells of telencephalic neuroepithelium where it modulates both progenitor cell proliferation and differentiation. FOXG1B is also found in the adult brain located in structures originating from the telencephalon including the cerebral cortex, hippocampus, olfactory bulbs and the basal ganglia (Dou et al., 1999; Hanashima et al., 2002). Furthermore, this gene is thought to act as an oncogenic transformer and transcriptional repressor through specific interactions with Groucho, Hes and Smad proteins (Yao et al., 2001; Rodriguez et al., 2001). More importantly, *FOXG1B* has been proposed as a major FoxO Forkhead transcription factor that acts as a signal transducer between the Smad, PI3K and FoxG1B pathways. The activity of these pathway interactions has been shown to be involved in the resistance to TGF-β mediated cytostasis during telencephalic neuroepithelium development and in glioblastoma brain tumour cells (Seoane et al., 2004).

TGF-β induced cytostasis requires the transcriptional activation of cyclin dependent kinase inhibitors *CDKN1A* and *CDKN2B* and repression of growth-promoting factors *c-myc* and *Id1-Id3* to mediate cell cycle arrest. In this study, *CDKN1A* and *Id1*, *Id2* and *Id3* were significantly up-regulated and *CDKN2B* was significantly down-regulated (no data was available for c-myc). Seoane et al. have shown that *FOXG1B* is an antagonist that suppresses *CDKN1A* induction by binding to and inhibiting the FoxO-Smad activator complex. Loss of this gene in PAI may therefore prevent *CDKN1A* transcriptional inhibition promoting TGF-β induced cytostasis (Seoane et al., 2004).

The involvement of *FOXG1B* in PI3K (the pathway most affected by differential gene expression in PAI) highlights the impact *FOXG1B* disruption may have on intracellular

signalling. The location of this gene in a region of LCV at 14q12 also suggests that some individuals may be predisposed to PAI development.

CNAs of individual clones at 9p21.2-p23, 10q26.3, 12q24.33, and 17q21.31 and a cluster of 7 clones at 7q11.23 correlated with differential gene expression. Two genes *BCL7B* and *BCL7A* located at 7q11.23 and 12q24.33 respectively are from the same family and share 90% homology. *BCL7B* maps to a region deleted in the congenital disorder Williams syndrome, although little is known about its function (Jadayel et al., 1998). *BCL7A* is a putative tumour suppressor gene which is hypermethylated in primary cutaneous T-cell lymphoma (CTCL) (van Doorn et al., 2005). Earlier studies have demonstrated the involvement of the *BCL7A* locus in a recurrent break point in B-cell lymphomas and a complex translocation and rearrangement in specific cell lines (Zani et al., 1996). Expression profiling of mycosis fungoides (MF), a common CTCL, and B-cell lymphoma identified the down-regulation of *BCL7A* as part of a molecular signature that could distinguish MF, inflammatory dermatoses (ID) and distinct types of diffuse large B-cell lymphoma (Alizadeh et al., 2000; Tracey et al., 2003). The loss of *BCL7A* has also been associated with disrupted NF-kappaB signalling and unfavourable prognosis in patients with B-cell lymphomas, suggesting that this gene has tumour suppressor properties (Martinez-Delgado et al., 2004).

Reduction of *SH3GL2* expression correlated with loss at 9p21.2-p23. A recent study indicates that *SH3GL2* is involved in the development and progression of laryngeal squamous cell carcinoma (LSCC), as expression levels correlate with pathological classification (Shang et al., 2007). The expression of *SH3GL2* also correlates with distinct stages of intestinal-type gastric cancer, although a functional role in tumourigenesis has not yet been determined (Sun et al., 2006).

TUBG2, located at 17q21.31, shares 97.3% amino acid identity with *TUBG1* and the two genes are co-expressed in a variety of tissues. This suggests that the functions of *TUBG1* and *TUBG2* are similar and both have a significant role in the organization of the microtubule cytoskeleton (Wise et al., 2000). *CNTNAP1*, also located at 17q21.31, is transcribed predominantly in brain and the architecture of the protein extracellular domain is similar to that of neurexins. *CNTNAP1* plays an important role in the creation and maintenance of the paranodal region of mylinated axons, enabling recruitment and

activation of intracellular signalling pathways in neurons (Geurts et al., 2003). *DRD1IP* maps to 10q26.3 and encodes a brain-specific dopamine receptor 1-interacting protein involved calcium signalling (Laurin et al., 2005). However, a role for these two genes in tumour development has yet to be established

Promoter hypermethylation in paediatric pilocytic astrocytoma biopsies

Down-regulation of gene expression through promoter hypermethylation is an important mechanism of transcriptional silencing in many tumours. Hypermethylation of the six genes investigated in this study has been detected in more than 30% of several tumour types including gastric carcinoma, lung cancer and renal cell carcinoma Moreover, *CCNA1* was hypermethylated in 100% of colorectal adenoma (Kikuchi et al., 2002; Toyooka et al., 2003; Oshimo et al., 2004; Xu et al., 2004; Morris et al., 2005; Takahashi et al., 2005). All six genes showed a 2-fold and significant down-regulation in PAI compared to normal brain but hypermethylation of gene promoter regions was not found. Only one previous study has investigated hypermethylation in paediatric PAI involving *MGMT*, *GSTP1*, *DAPK1*, $p14^{ARF}$, *THBS-1*, *TIMP-3*, *TP73*, *CDKN2A*, *RB-1* and *TP53*. Hypermethylation of *CDKN2A* was most frequent occurring in 46% of cases. However, 5 of the remaining genes were hypermethylated in less than 8% of cases (Gonzalez-Gomez et al., 2003b). *CDKN2A* hypermethylation was not investigated in this study as this gene was not found to be significantly down-regulated in the 19 PAI investigated. It is possible that methylation is not the predominant mechanism of gene silencing in PAI development and other epigenetic processes such as histone modification and microRNAs may play a more significant role.

Overall, differential gene expression in PAI can be linked to functions promoting tumourigenesis. In the PAI studied here, the differential expression of several genes was also associated with functions that may hinder tumour progression and could be attributed to the benign behaviour of this tumour. CNAs of whole chromosomes or arms were not found in these tumours, suggesting that large CNAs are not involved in the development of paediatric PAI. However, specific correlations were identified between single clone alterations and differential expression. These alterations were often located in regions of LCVs, suggesting that small regions of alteration may predispose individuals to tumour development. The investigation of the methylation status of a

small number of genes was completed in this study, but larger more extensive studies are needed to determine the involvement of this mechanism in PAI development.

CHAPTER 5

DIFFERENTIAL GENE EXPRESSION AND COPY NUMBER ALTERATIONS IN PAEDIATRIC ASTROCYTOMA SHORT-TERM CELL CULTURES

INTRODUCTION

Histopathologically, paediatric astrocytoma resemble corresponding grades of adult astrocytoma. However, studies have shown that their biological and clinical behaviour is different to that of their adult counterparts, suggesting that these tumours have a different molecular pathogenesis.

Cytogenetic analysis of paediatric PAI has shown that the majority of these tumours (>70%) have a normal karyotype (Bhattacharjee et al., 1997). Numerical and structural abnormalities of chromosomes 5, 6, 7, 8 and 9 have been reported, although definitive, non-random aberrations have not yet been identified (White et al., 1995; Zattara-Cannoni et al., 1998; Shlomit et al., 2000; Sanoudou et al., 2000b; Rickert and Paulus, 2004).

In DAII, AAIII and GBMIV of patients below 15 years of age there is a correlation between karyotype complexity and tumour grade. DAII often have a normal karyotype or show simple chromosome changes compared to high-grade astrocytoma that show multiple complex chromosome aberrations frequently displaying hyperdiploid or polypoid karyotypes (Agamanolis and Malone, 1995; Bigner et al., 1997; Bhattacharjee et al., 1997; Roberts et al., 2001). Studies investigating diffuse astrocytoma in children show inconsistent findings. Specific studies describe chromosome alterations that are common in adult astrocytoma including -17p, +7, -10 and -22 (Schrock et al., 1996; Bhattacharjee et al., 1997). Other studies describe different alterations to those seen in adults (Neumann et al., 1993; Agamanolis and Malone, 1995; Cheng et al., 1999).

Various studies previously discussed have investigated the expression of specific genes in grades of paediatric astrocytoma or genome wide gene expression in individual grades of paediatric astrocytoma. No studies to date have included genome wide expression profile analysis of all grades of paediatric astrocytoma.

The aims of this Chapter were to investigate genome wide gene expression in paediatric astrocytoma short-term cell cultures in order to identify cellular pathways affected by differential gene expression, correlate these changes with CNAs as a possible mechanism and identify any specific gene signatures that correspond to specific tumour or patient parameters including tumour grade, location, patient survival, age or sex. Moreover, the inclusion of 7 adult GBMIV short-term cell cultures has allowed a comparison to be made between the expression profiles of these tumours and those from paediatric patients. This chapter also aims to confirm that astrocytoma short-term cell cultures are representative of the tumour of origin and that conclusions drawn from these cultures regarding CNAs and gene expression are comparative to those made using biopsy samples.

The Affymetrix GeneChip® U133A array was used to generate gene expression profiles and identify differentially expressed genes in 35 paediatric astrocytoma short-term cell cultures derived from 19 PAI, 2 DAII, 3 AAIII and 11 GBMIV according to the 2007 WHO grading scheme. It was not possible to review the diagnosis of every case according to the 2007 WHO classification guidelines including 2 DAII and 5 GBMIV (Refer to Table 2.2). In addition, 6 of these short-term cell culture samples were previously analysed in Chapter 3, IN1740, IN2110, IN2356, IN2368, IN2921 and IN3013. The aCGH SpectralChip™ 2600 array was used to define genomic CNAs in 32 of these tumours; sufficient DNA was not available for tumours IN1740, IN1930 and IN2087.

The experimental analysis was carried out as previously described and the mean expression of each gene in all samples was used for the per gene normalisation. The controls included in this study were 2 cultured foetal astrocyte samples (details of these samples can be found in Table 2.1 and Chapter 3). Due to the small number of controls and the foetal origin as discussed in Chapter 3, the experiment was not normalised to the controls as previously carried out in Chapter 4. However, the normal foetal astrocytes were included in the study so that some comparisons could be made between the expression profiles of these samples and those of the paediatric astrocytomas short-term cell cultures. Gene probe sets present in 10 of the 37 samples (11491 after cleaning) were included in the experimental analysis. This sample number was felt to be

appropriate given that 11 GBMIV (according to the 2007 WHO grading scheme) composed the smallest sample group categorised by grade. This allowed those genes that may have been down-regulated in a specific grade and consequently detected as absent on the array, to be included in further analysis.

To compare the paediatric short-term cell cultures to those derived from adult tumours, 7 adult GBMIV short-term cultures were added to the experiment and the hierarchical approach repeated (details of these samples can be found in Table 2.3). Three of these samples were previously analysed in Chapter 3, IN2435, IN2731 and IN2810. All experiment parameters were kept the same and the foetal astrocytes were removed from the experiment, as the comparison was only between the tumour samples.

Patient details of the tumour derived short-term cell cultures can be found in Tables 2.2 and 2.3. The mean paediatric patient age was 8.4 years, ranging from 0.2 years to 15.9 years. At the time of this study 22 patients were alive. According to the 2007 WHO grading scheme, of the19 PAI patients 2 are deceased (survival 12 and 60 months), 5 had been discharged from hospital and were no longer being monitored (follow-up between 18-125 months) and 12 patients had a follow-up between 13-160 months. Of the 2 DAII patients 1 was deceased (survival 92 months) and 1 had a follow-up of 60 months. Of the 3 AAIII patients, 2 were deceased (survival 12 and 14 months) and 1 had a follow-up of 53 months. Of the 10 GBMIV patients 5 were deceased (survival between 3-33months), 1 had been discharged, 3 had a follow-up between 7-80 months and survival data for 1 patient was not available. Survival data and follow-up was limited for the adult patients, the mean patient age at diagnosis was 60.4 years and ranged from 51 years to 70 years. All adult short-term cell cultures were derived from adult primary GBMIV biopsies.

The survival data of patients in this study was considered to be unreliable as unform treatment regimes were not applied to the patients. Consequently, although survival was not statistically analysised is was considered with caution during some analysis approaches.

RESULTS

Gene expression profiles of paediatric astrocytoma short-term cell cultures

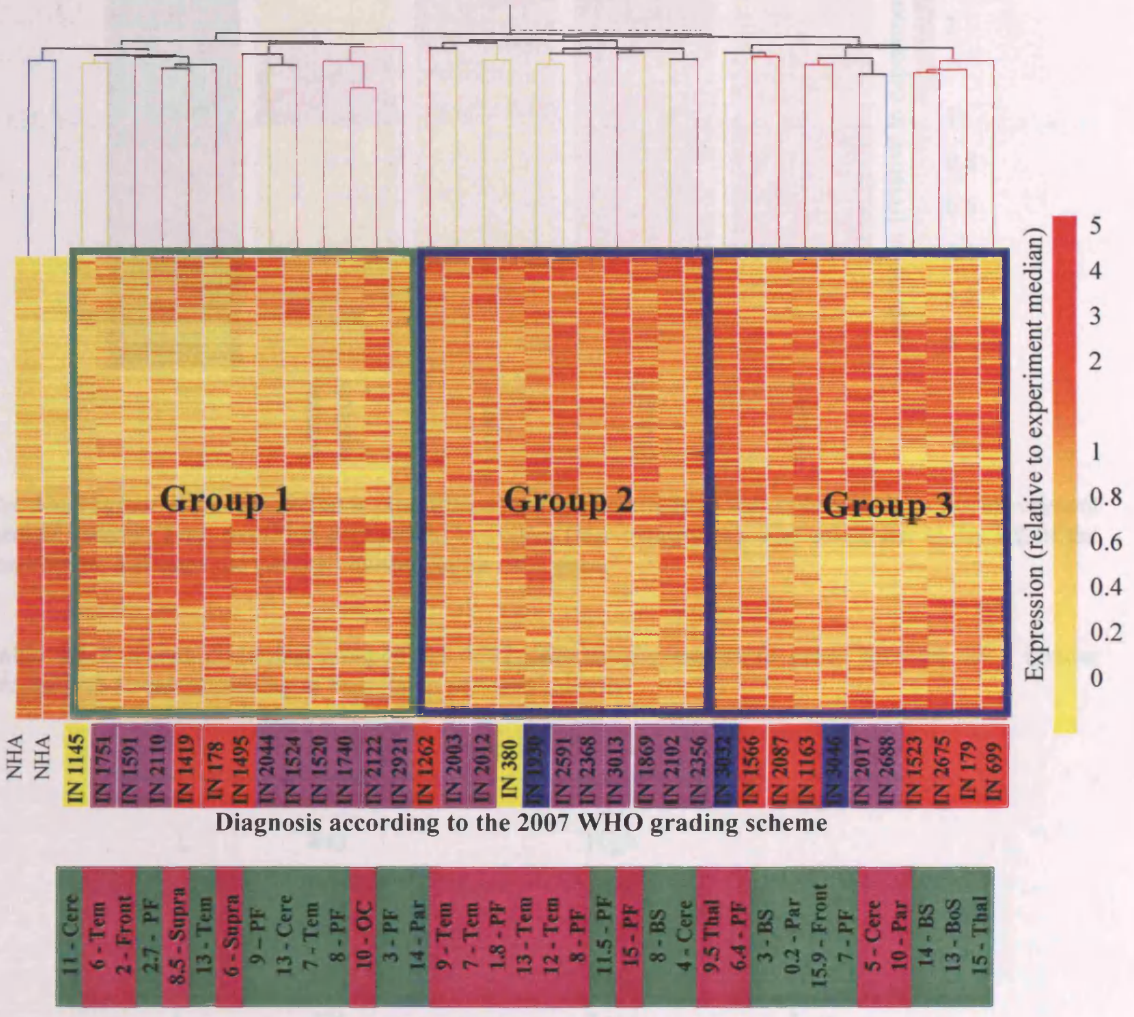
Using the Affymetrix GeneChip® U133A array the expression pattern of approximately 22000 genes were investigated in 35 paediatric astrocytoma short-term cell cultures of various grades and 2 normal human cultured foetal astrocytes. Unsupervised hierarchical clustering using 11491 reliable gene expression results was used to generate a dendrogram illustrating the similarity in expression profiles of the paediatric astrocytoma short-term cell cultures (Figure 5.1). The tumour cultures do not group or order according to tumour location or patient age or sex. However, the cultures do cluster into 3 clear groups. According to the 2007 WHO grading scheme, 2 groups are comprised of predominately PAI short-term cell cultures with several high-grade cultures and the third group consists of high-grade short-term cell cultures and 2 PAI cultures (Figure 5.1). The 3 culture cluster groups will be referred to as Group 1, Group 2 and Group 3.

The PAI short-term cell cultures of this study appear to cluster into 2 clear groups as seen by Wong et al. (2005) and discussed in detail in Chapter 4. These cluster groups are Groups 1 and 2 according to the analysis completed in the rest of Chapter 5. The expression pattern of the 13 reliable gene expression results that correspond to 13 of the 89 genes used by Wong et al. (2005) to characterise two PAI sub-groups was investigated in the tumour cultures of this experiment. Differential gene expression was not seen between the short-term cell culture Groups or normal controls (data not shown). Furthermore, Wong et al. (2005) identified the biological processes cell adhesion, regulation of cell growth, cell motility, nerve ensheathment and angiogenesis as showing significant differential gene expression between the two PAI sub-groups. In this experiment, only cell adhesion and angiogenesis showed a significant difference between the 2 PAI Groups.

From the group of 11491 gene probe sets, 4682 were identified that showed a 2-fold and significant difference in expression between the 3 Groups and the normal astrocyte controls. To generate molecular profiles of each tumour culture Group and identify genes that show similar and different expression patterns, the 4682 differentially

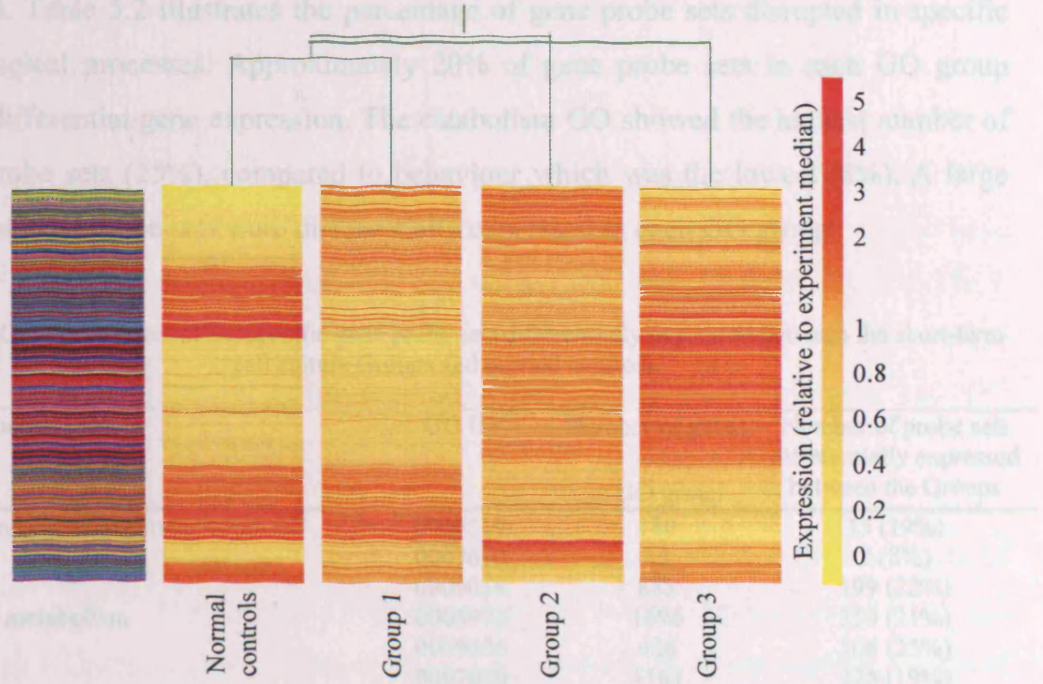
expressed genes were studied using k-means clustering. The genes were clustered into 10 sets according to expression pattern similarity (Figure 5.2 and Table 5.1).

Figure 5.1 Unsupervised hierarchical clustering of thirty-five paediatric astrocytoma short-term cell culture samples and two normal foetal astrocyte controls using 11491 reliable gene expression results.



In total, 4682 genes showed a 2-fold and significant change in expression between the 3 Groups and the normal astrocytes, these colour the dendrogram. The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median and ranges from red (high-expression) to yellow (low-expression). PAI = purple; DAII = yellow; Low-grade tumour = light-blue; AAIII = dark blue; GBMIV = red (according to the 2007 WHO grading scheme). The green and pink boxes below the dendrogram labels represent the patient sex; male and female respectively. The age of the patient at diagnosis and tumour location are given within the coloured boxes. The abbreviations used are as follows; Cere = cerebellum, Tem = temporal, Front = frontoparietal, Supra = supratentorial, PF = posteria fossa, OC = Optic Chiasm, Par = parietal, BS = brain stem, Thal = thalamus and BoS = base of skull.

Figure 5.2 The dendrogram illustrates the expression pattern of the 4682 differentially expressed probe sets, between the 3 culture Groups and the normal control.



The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median, ranging from red (high-expression) to yellow (low-expression). The colour bar to the left of the dendrogram indicates the k-means cluster set for each gene.

Table 5.1 K-means clustering grouped the 4682 differentially expressed genes between the 3 tumour Groups into 10 sets according to expression pattern similarity.

Set No.	No. of genes	Group 1	Group 2	Group 3
1	723			High
2	443		High	
3	362	Low	Low	
4	541		High	High
5	665		High	High
6	776		Low	Low
7	298	Low	Low	Low
8	388	High	High	
9	250		Low	
10	182	Low	Low	Low

The Table indicates high or low gene expression in specific tumour Groups compared to the remaining Groups and normal controls.

K-means cluster sets 4 and 5, and sets 7 and 10 show the same expression pattern between the 3 tumour Groups and the normal controls. The remaining sets show expression patterns that can be used to distinguish the 3 tumour Groups.

To characterise the 4682 probe sets, those involved in specific biological processes were identified. Table 5.2 illustrates the percentage of gene probe sets disrupted in specific GO biological processes. Approximately 20% of gene probe sets in each GO group showed differential gene expression. The catabolism GO showed the highest number of altered probe sets (25%), compared to behaviour which was the lowest (8%). A large number of gene probe sets were differentially expressed in each GO group.

Table 5.2 Gene ontologies of the specific gene probe sets differentially expressed between the short-term cell culture Groups and normal controls.

Biological process	GO ID	Number of genes in GO group	Number of probe sets differentially expressed between the Groups
Amino acid and derivative metabolism	0006519	180	35 (19%)
Behaviour	0007610	83	7 (8%)
Biosynthesis	0009058	885	199 (22%)
Carbohydrate metabolism	0005975	1696	350 (21%)
Catabolism	0009056	426	106 (25%)
Cell cycle	0007049	1161	225 (19%)
Cell death	0008219	863	144 (17%)
Cell differentiation	0030154	1657	278 (17%)
Cell growth	0016049	3993	673 (17%)
Cell homeostasis	0019725	90	18 (20%)
Cell proliferation	0008283	1067	211 (20%)
Cell recognition	0008037	1820	301 (17%)
Cell-cell signalling	0007267	2582	443 (17%)
Chemi-mechanical coupling	0006943	102	11 (11%)
Co-enzyme and prosthetic group metabolism	0006731	1163	192 (17%)
Cytoskeleton organisation and biogenesis	0007010	1536	314 (20%)
DNA metabolism	0006259	1470	256 (17%)
Electron transport	0006118	3286	560 (17%)
Embryonic development	0009790	779	117 (15%)
Energy pathways	0006091	1497	291 (19%)
Growth	0040007	1405	270 (19%)
Host-pathogen interactions	0030383	4051	602 (15%)
Ion transport	0006811	2474	428 (17%)
Lipid metabolism	006629	859	183 (21%)
Mitochondria organisation and biogenesis	0007005	1273	262 (21%)
Morphogenesis	0009653	301	43 (14%)
Protein biosynthesis	0006412	1060	231 (22%)
Protein modification	0006412	307	56 (18%)
Protein transport	0015031	2031	456 (22%)
Regulation of gene expression; epigenetic	0040029	3969	698 (18%)
Reproduction	0000003	141	17 (12%)
Response to endogenous stimuli	0009719	103	21 (20%)
Response to stress	0006950	427	71 (17%)
Signal transduction	0007165	4158	647 (16%)
Transcription	0006350	3697	563 (15%)
Unknown biological processes	0000004	11034	950 (8%)

The percentage of gene probe sets in each ontology group that shows differential expression between the short-term cell culture Groups and normal controls is also shown in brackets.

To further characterise the differences in expression profile between the 3 tumour culture Groups and normal controls, the 4682 probe sets were divided into clusters that showed a 2-fold and significant difference in expression between each tumour culture Group and the normal control. In total, 1764 probe sets were differentially expressed between Group 1 and the controls, 3035 between Group 2 and the controls and 3375 between Group 3 and controls. The biological processes influenced by differential gene expression in each Group were investigated using Onto-tools (Tables 5.3-5.5). The p value for each process is the probability that deregulated genes are associated with a function by chance and is based on a binomial probability distribution and GO annotations.

The most common biological processes significantly altered in the tumour cultures of Groups 1, 2 and 3 compared to the normal controls involve antigen processing and presentation. The biological processes of Group 3 involve more input genes compared to Groups 1 and 2. Furthermore, Onto-tools groups similar process under one title if all are significantly altered. For example, signal transduction is significantly altered in Group 3 compared to Groups 1 and 2, but this includes negative regulation of lipopolysaccharide-mediated signalling pathway, positive regulation of transforming growth factor beta receptor signalling pathway, negative regulation of Wnt receptor signalling pathway, Notch signalling pathway, positive regulation of I-kappaB kinase/NF-kappaB cascade and positive regulation of vascular endothelial growth factor receptor pathway, identified in Groups 1 and 2, as well as G-protein coupled receptor signalling and small GTPase signalling that were not significantly altered in Groups 1 and 2. Furthermore, cell differentiation biological processes were frequently disrupted in the tumour cultures of Group 1 compared to Groups 2 and 3.

Table 5.3 Significantly altered biological processes in the Group 1 short-term cell cultures.

Biological Process	Gene input	p value
Cell adhesion	101 (5.73%)	3.0 E-5
Organ morphogenesis	30 (1.7%)	3.0 E-5
Antigen processing, endogenous antigen via MHC class I	17 (0.96%)	1.6 E-4
Muscle development	25 (1.42%)	1.6 E-4
Antigen presentation, endogenous antigen	14 (0.79%)	4.7 E-4
Glutathione biosynthesis	6 (0.34%)	0.006
Germ cell migration	5 (0.28%)	0.006
Antigen presentation	13 (0.74%)	0.006
Cell growth	16 (0.91%)	0.006
Prostaglandin biosynthesis	7 (0.4%)	0.006
Cell recognition	6 (0.34%)	0.008
Regulation of cell migration	8 (0.45%)	0.008
Regulation of cell shape	13 (0.74%)	0.008
Mevalonate transport	3 (0.17%)	0.012
Regulation of circadian sleep/wake cycle	3 (0.17%)	0.012
Actomyosin structure organisation and biogenesis	4 (0.23%)	0.014
Angiogenesis	15 (0.85%)	0.027
mRNA stabilisation	3 (0.17%)	0.027
Negative regulation of chemokines biosynthesis	3 (0.17%)	0.027
Sulfate assimilation	3 (0.17%)	0.027
Deoxribonuclease diphosphate metabolism	2 (0.11%)	0.034
Fatty acid biosynthesis	11 (0.62%)	0.034
Leukocyte adhesive activation	2 (0.11%)	0.034
Negative regulation of lipopolysaccharide-mediated signalling pathway	2 (0.11%)	0.034
Neuron lineage restriction	2 (0.11%)	0.034
Positive regulation of calcium-independent cell-cell adhesion	2 (0.11%)	0.034
Positive regulation of transforming growth factor beta receptor signalling pathway	2 (0.11%)	0.034
Regulation of endothelial cell differentiation	2 (0.11%)	0.034
Response to toxin	5 (0.28%)	0.034
Endothelial cell differentiation	3 (0.17%)	0.036
Fat cell differentiation	5 (0.28%)	0.036
Keratinocyte differentiation	5 (0.028%)	0.036
Positive regulation of translation	3 (0.17%)	0.036
Regulation of innate immune response	3 (0.17%)	0.036
Vasculogenesis	5 (0.28%)	0.036
Myoblast differentiation	4 (0.23%)	0.037
Negative regulation of transcription factor activity	4 (0.23%)	0.037
Negative regulation of Wnt receptor signalling pathway	4 (0.23%)	0.037
Protein export from nucleus	4 (0.23%)	0.037
Positive regulation of cell proliferation	25 (1.42%)	0.038
Cell fate determination	5 (0.28%)	0.049
Cholesterol homeostasis	4 (0.23%)	0.049
Cortical actin cytoskeleton organisation and biogenesis	5 (0.28%)	0.049
Membrane protein ectodomain proteolysis	5 (0.28%)	0.049
Organic anion transport	6 (0.34%)	0.049
Phosphate transport	17 (0.96%)	0.049
Regulation of cell proliferation	5 (0.28%)	0.049
Regulation of embryonic development	4 (0.23%)	0.049
Negative regulation of cell proliferation	32 (1.81%)	0.049
Notch signalling pathway	9 (0.51%)	0.049

The gene input percentage indicates the number of genes involved in the specific biological process as a percent of the overall input, in this case 1764.

Table 5.4 Significantly altered biological processes in the Group 2 short-term cell cultures.

Biological Process	Gene input	p value
Antigen presentation, endogenous antigen	20 (0.66%)	2.7 E -4
Antigen processing, endogenous antigen via MHC class I	23 (1.76%)	2.7 E -4
Cell Adhesion	135 (4.45%)	2.7 E -4
Lipid metabolism	71 (2.34%)	9.4 E -4
Antigen presentation	20 (0.66%)	0.001
Positive regulation of I-kappaB kinase/NF-kappaB cascade	36 (1.19%)	0.003
Superoxide metabolism	9 (0.3%)	0.010
Cell-matrix adhesion	25 (0.82%)	0.014
Germ cell migration	6 (0.2%)	0.014
Organ morphogenesis	34 (1.12%)	0.014
Attachment of GPI anchor protein	5 (0.16%)	0.014
ER to golgi vesicle-mediated transport	30 (0.99%)	0.018
Electron transport	81 (2.67%)	0.018
Fatty acid desaturation	6 (0.2%)	0.020
Induction of positive chemotaxis	6 (0.2%)	0.020
Barbed-ended actin filament capping	15 (0.49%)	0.026
Angiogenesis	22 (0.72%)	0.026
Positive regulation of vascular endothelial growth factor receptor pathway	4 (0.13%)	0.030
Phosphate transport	27 (0.89%)	0.039
Cell motility	42 (1.28%)	0.046
Cell growth	20 (0.66%)	0.048

The gene input percentage indicates the number of genes involved in the specific biological process as a percent of the overall input, in this case 3035.

Table 5.5 Significantly altered biological processes in the Group 3 short-term cell cultures.

Biological Process	Gene input	p value
ER to Golgi vesicle-mediated transport	42 (1.24%)	4.0 E -5
Protein biosynthesis	100 (2.96%)	6.0 E -5
Nuclear mRNA splicing, via spliceosome	62 (1.84%)	5.5 E -5
Antigen presentation, endogenous antigen	20 (0.59%)	0.001
Antigen processing, endogenous antigen via MHC class I	22 (0.65%)	0.002
Protein transport	91 (2.7%)	0.002
Tricarboxylic acid cycle	18 (0.53%)	0.005
Antigen presentation	19 (0.56%)	0.011
Signal transduction	200 (5.93%)	0.019
Ion transport	38 (1.13%)	0.039
Protein targeting	20 (0.59%)	0.041
Cell redox homeostasis	6 (0.18%)	0.044
Electron transport	86 (2.55%)	0.047
Mitochondrial electron transport, NADH to uniquinone	10 (0.3%)	0.047
Response to unfolded protein	20 (0.59%)	0.047

The gene input percentage indicates the number of genes involved in the specific biological process as a percent of the overall input, in this case 3375.

To further investigate the overall gene expression profiles of each tumour culture Group and correlate changes in biological pathways to tumour development, the Onto-tools software was used to rank KEGG pathways using the 4682 differentially expressed gene probe sets. In total, 53 pathways were ranked, only those of interest are displayed in Table 5.6. The focal adhesion and extracellular matrix receptor interaction pathways

were ranked first and second in all 3 tumour culture Groups. The Wnt signalling pathway and the cell cycle showed large differences in rank between the 3 Groups (Table 5.6). These pathways were investigated in more detail as disruption of the cell cycle is known to be involved in tumour development and Wnt signalling is involved in foetal development and control of stem cell differentiation (Clark et al., 2007). It was hypothesized that the disruption of these pathways through differential gene expression occurs in each Group, but to different extents. With the number of differentially expressed genes being greater in the more malignant tumour cultures of Group 3.

The genes involved in Wnt signalling pathway and cell cycle show significant changes in expression between the 3 tumour Groups (Figures 5.3 and 5.4). The Wnt signalling pathway in Group 1 tumour cultures has fewer differentially expressed genes compared to Group 2 and 3 tumour cultures. Furthermore, the majority of genes in the Wnt signalling pathway are up-regulated in Groups 2 and 3 compared to Group 1. The signalling of this pathway is further altered by the down-regulation of APC expression and up-regulation of β-catenin in Group 3 tumour cultures only (Figure 5.3).

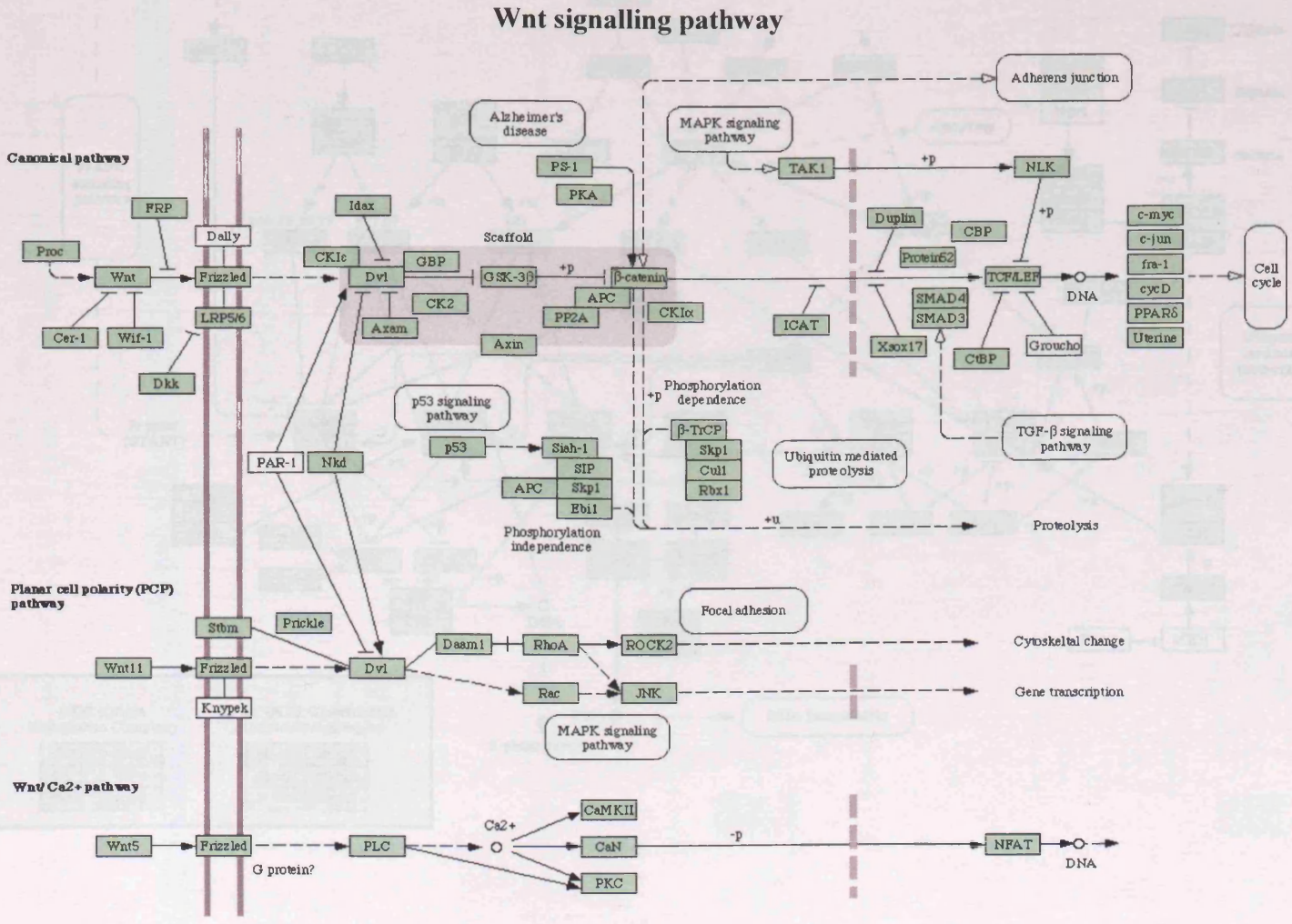
The majority of genes involved in the cell cycle of Group 3 tumour cultures show up-regulation, including *TP53*, genes of the APC/C complex and mini chromosome maintenance (MCM) genes. In Group 2 tumour cultures *TP53* is also up-regulated but genes of the APC/C complex are down-regulated. Key cell cycle components *CCNE*, *CCNA* and *CDK2* also show down-regulation in Group 2 tumour cultures compared to Groups 1 and 3. Moreover, *TP53* and genes of the APC/C complex are not differentially expressed in Group 1 tumour cultures (Figure 5.4).

Table 5.6 The Onto-tools result ranking the KEGG pathways using the 4682 differentially expressed probe sets for each of the 3 tumour Groups.

Rank	Pathway Name	Impact Factor	%Pathway Genes in Input	Rank	Pathway Name	Impact Factor	%Pathway Genes in Input	Rank	Pathway Name	Impact Factor	%Pathway Genes in Input
1	Focal adhesion	13.24	21.65	1	ECM-receptor interaction	12.70	35.63	2	ECM-receptor interaction	8.57	34.48
2	ECM-receptor interaction	11.31	25.29	2	Focal adhesion	9.86	28.35	3	Focal adhesion	7.82	28.87
8	TGF-beta signalling pathway	6.35	17.86	4	Colorectal cancer	6.90	32.47	4	Colorectal cancer	6.96	33.77
14	Regulation of actin cytoskeleton	5.14	15.05	6	**Wnt signalling pathway**	5.51	24.49	5	Phosphatidylinositol signalling system	6.74	16.46
20	Antigen processing and presentation	4.26	13.95	10	TGF-beta signalling pathway	4.85	23.81	6	**Wnt signalling pathway**	5.59	25.85
21	Phosphatidylinositol signalling system	4.22	12.66	11	Regulation of actin cytoskeleton	4.49	22.33	11	**Cell cycle**	4.79	25.00
22	Notch signalling pathway	3.63	17.39	17	Antigen processing and presentation	4.04	19.77	17	Antigen processing and presentation	3.98	23.26
23	MAPK signalling pathway	3.38	12.82	19	MAPK signalling pathway	3.83	20.88	22	mTOR signalling pathway	3.22	28.57
25	**Wnt signalling pathway**	3.31	13.61	21	Calcium signalling pathway	3.43	18.75	25	TGF-beta signalling pathway	3.01	22.62
30	Colorectal cancer	2.94	15.58	22	Notch signalling pathway	3.38	23.91	28	Apoptosis	2.83	20.24
32	Calcium signalling pathway	2.76	11.93	31	**Cell cycle**	2.82	21.43	29	Regulation of actin cytoskeleton	2.81	20.39
36	Apoptosis	2.38	13.10	36	Jak-STAT signalling pathway	2.38	18.95	35	MAPK signalling pathway	2.32	19.41
40	Jak-STAT signalling pathway	2.19	11.11	37	mTOR signalling pathway	2.22	20.41	36	Notch signalling pathway	2.23	17.39
43	Hedgehog signalling pathway	2.01	10.53	39	Hedgehog signalling pathway	2.17	19.30	39	Hedgehog signalling pathway	1.98	14.04
46	mTOR signalling pathway	1.91	12.25	42	Phosphatidylinositol signalling system	2.07	20.25	43	Calcium signalling pathway	1.66	18.18
49	**Cell cycle**	1.70	11.61	43	Apoptosis	2.02	19.05	45	Jak-STAT signalling pathway	1.55	13.73

Group 1 = blue; Group 2 = green; Group 3 = red. The pathways are ranked according to impact factor, taking into account the fold change, the number of genes disrupted in a pathway and the influence each gene exerts on a pathway.

These are reference KEGG pathways for Figures 5.3 and 5.4, as the gene names are not always clear in these Figures.



Cell cycle

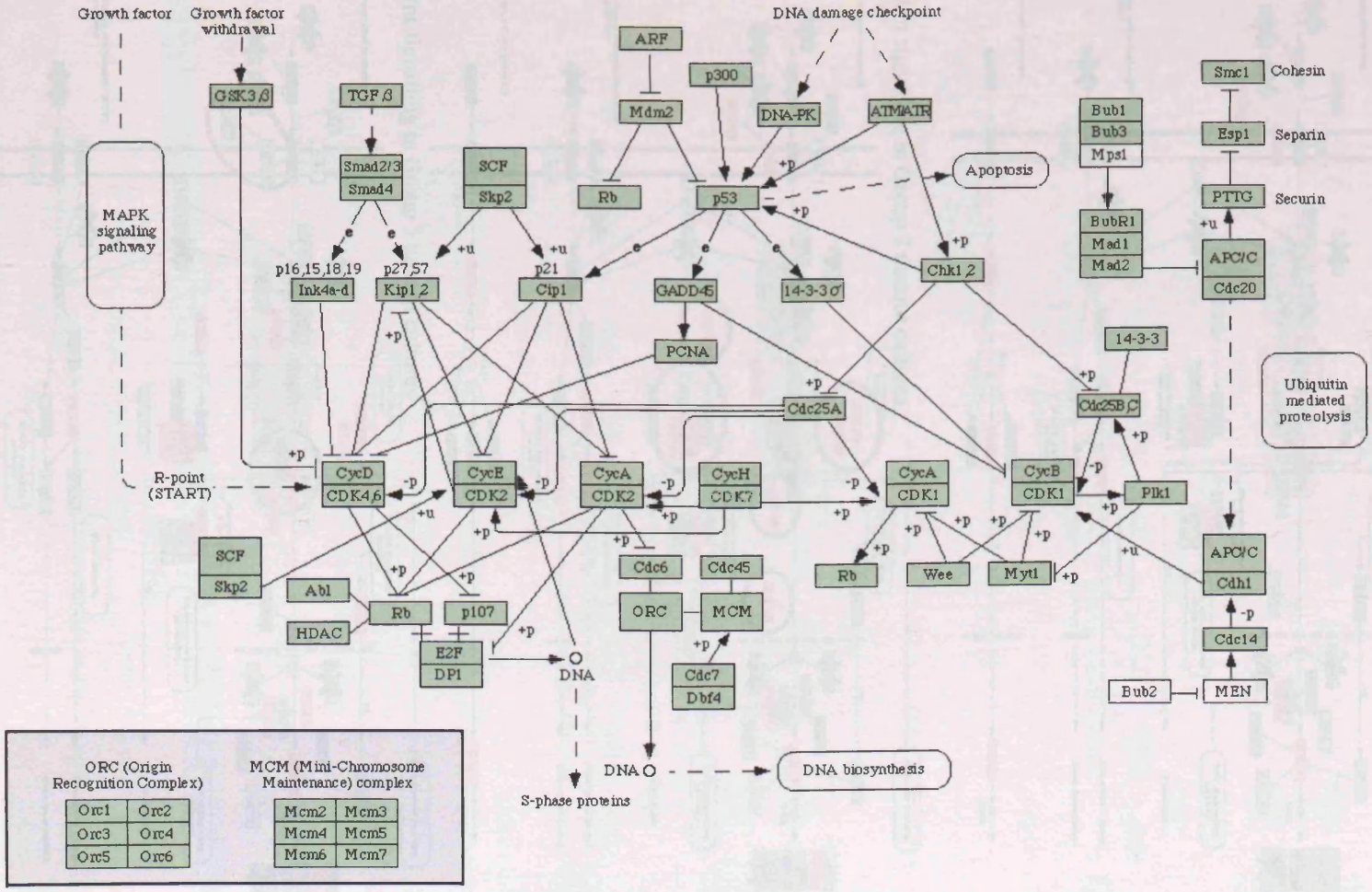
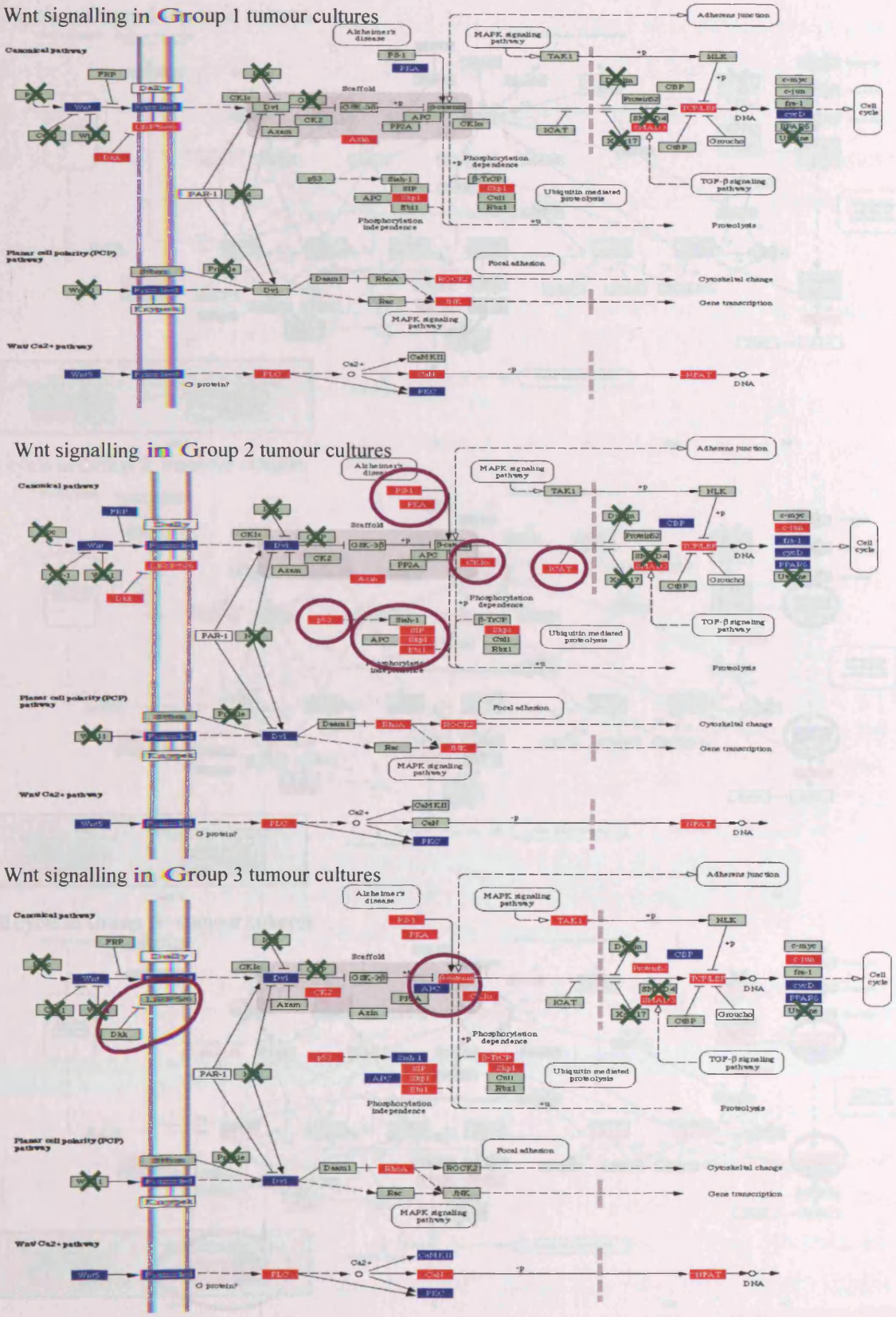

Growth factor
Growth factor withdrawal
GSK3β
TGFβ
MAPK signaling pathway
Smad2/3
Smad4
SCF
Skp2
p16,15,18,19
Ink4a-d
p27,57
Kip1,2
p21
Cip1
ARF
p300
Mdm2
DNA-PK
ATM/ATR
DNA damage checkpoint
Rb
p53
Apoptosis
GADD45
14-3-3σ
Chk1,2
PCNA
Cdc25A
Cdc25B,C
14-3-3
Bub1
Bub3
Mps1
BubR1
Mad1
Mad2
Smc1
Cohesin
Esp1
Separin
PTTG
Securin
APC/C
Cdc20
Ubiquitin mediated proteolysis
R-point (START)
CycD
CDK4,6
CycE
CDK2
CycA
CDK2
CycH
CDK7
CycA
CDK1
CycB
CDK1
Plk1
SCF
Skp2
Abl
HDAC
Rb
p107
E2F
DP1
DNA
Cdc6
Cdc45
ORC
MCM
Cdc7
Dbf4
Rb
Wee
Myt1
APC/C
Cdh1
Cdc14
Bub2
MEN
S-phase proteins
DNA
DNA biosynthesis
ORC (Origin Recognition Complex)
Orc1
Orc2
Orc3
Orc4
Orc5
Orc6
MCM (Mini-Chromosome Maintenance) complex
Mcm2
Mcm3
Mcm4
Mcm5
Mcm6
Mcm7

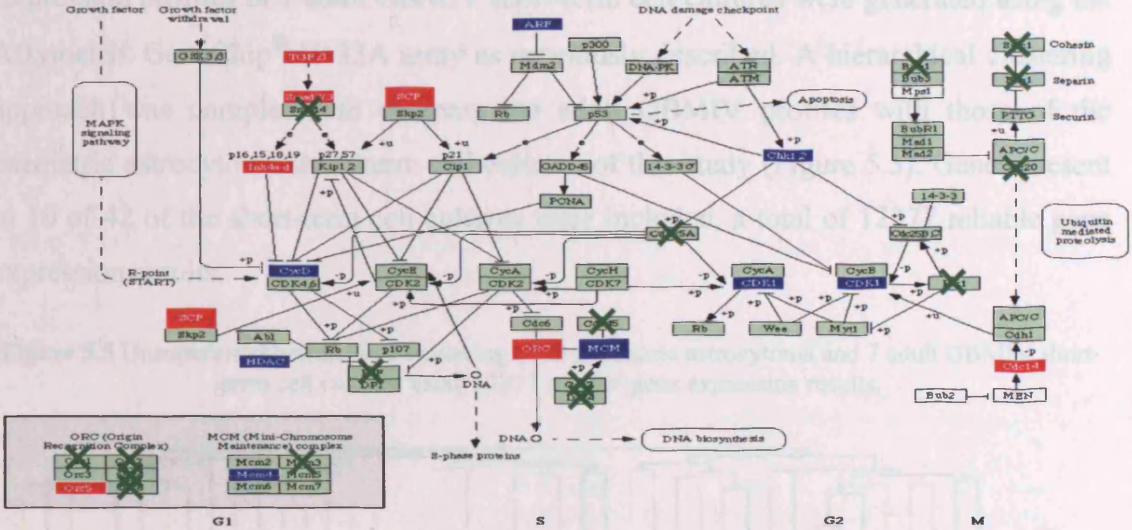
Figure 5.3 The differential gene expression found in the Wnt signalling pathway of Group 1, 2 and 3 tumour cultures.



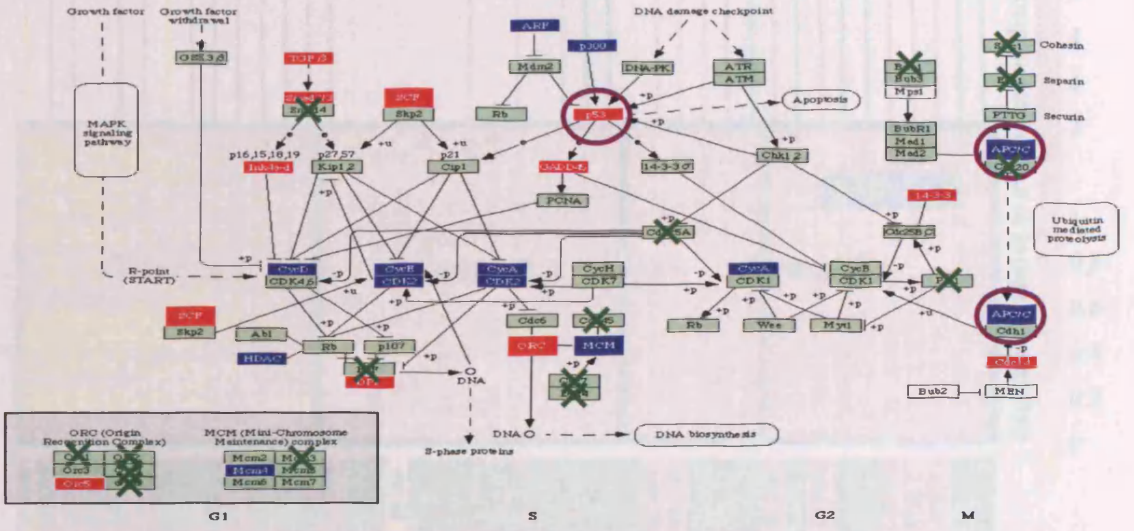
Up-regulated genes are red, down-regulated genes are blue, those in green showed no differential expression and reliable data was not available for genes indicated with a cross.

Figure 5.4 The differential gene expression found in the cell cycle signalling pathway of Group 1, 2 and 3 tumours.

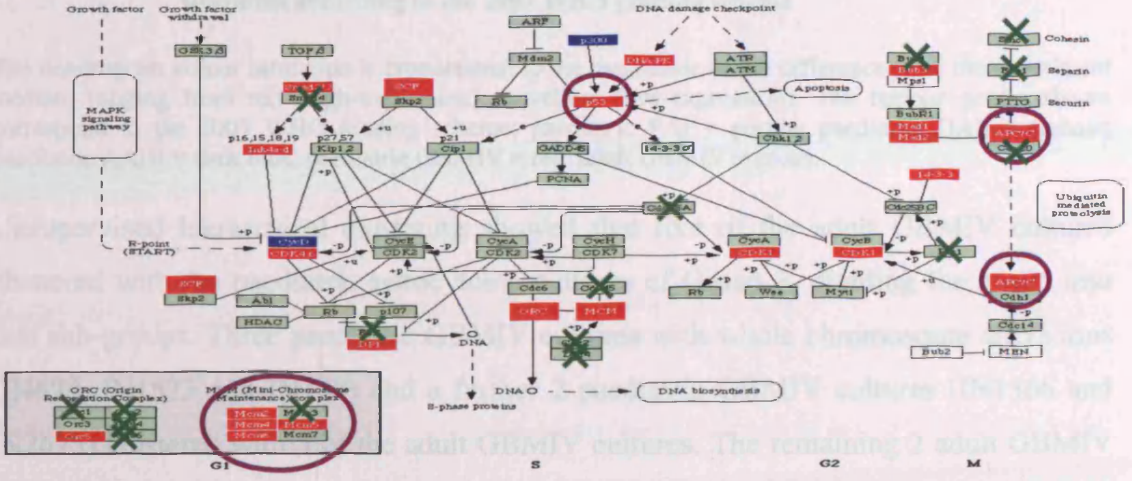
Cell cycle in Group 1 tumour cultures



Cell cycle in Group 2 tumour cultures



Cell cycle in Group 3 tumour cultures

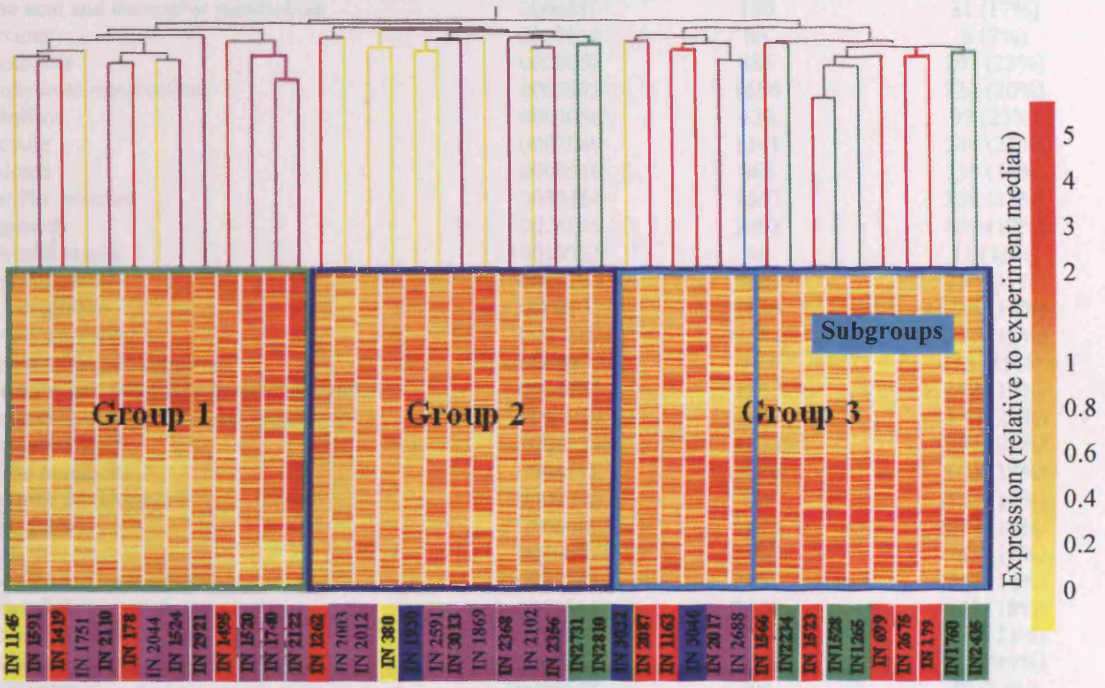


Up-regulated genes are red, down-regulated genes are blue, those in green showed no differential expression and reliable data was not available for genes indicated with a cross.

Comparison of adult GBMIV and paediatric astrocytoma short-term cell culture expression profiles

Expression profiles of 7 adult GBMIV short-term cell cultures were generated using the Affymetrix GeneChip® U133A array as previously described. A hierarchical clustering approach was completed to compare the adult GBMIV profiles with those of the paediatric astrocytoma short-term cell cultures of this study (Figure 5.5). Genes present in 10 of 42 of the short-term cell cultures were included, a total of 12377 reliable gene expression results.

Figure 5.5 Unsupervised hierarchical clustering of 35 paediatric astrocytoma and 7 adult GBMIV short-term cell cultures using 12377 reliable gene expression results.



The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median, ranging from red (high-expression) to yellow (low-expression). The tumour grades shown correspond to the 2007 WHO grading scheme; paediatric PAI = purple; paediatric DAII = yellow; paediatric AAIII = dark blue; paediatric GBMIV = red; adult GBMIV = green.

Unsupervised hierarchical clustering showed that five of the adult GBMIV cultures clustered with the paediatric astrocytoma cultures of Group 3, dividing the group into two sub-groups. Three paediatric GBMIV cultures with whole chromosome alterations (IN699, IN1523 and IN179) and a further 2 paediatric GBMIV cultures (IN1566 and IN2675) clustered with 5 of the adult GBMIV cultures. The remaining 2 adult GBMIV cultures clustered with the paediatric astrocytoma of Group 2.

From the group of 12377 gene probe sets, 4870 were identified that showed a 2-fold and significant difference in expression between the 3 Groups. Table 5.7 illustrates the percentage of gene probe sets disrupted in specific GO biological processes. Approximately 20% of gene probe sets in each GO group showed differential gene expression. The protein modification GO showed the highest number of altered genes (26%), compared to behaviour which was the lowest (7%).

Table 5.7 Gene ontologies of specific genes differentially expressed between the short-term cell culture Groups including the adult samples.

Biological process	GO ID	Number of genes in GO group	Number of genes differentially expressed between astrocytoma Groups
Amino acid and derivative metabolism	0006519	180	31 (17%)
Behaviour	0007610	83	6 (7%)
Biosynthesis	0009058	885	201 (23%)
Carbohydrate metabolism	0005975	1696	334 (20%)
Catabolism	0009056	426	97 (23%)
Cell cycle	0007049	1161	244 (21%)
Cell death	0008219	863	139 (16%)
Cell differentiation	0030154	1657	255 (15%)
Cell growth	0016049	3993	659 (17%)
Cell homeostasis	0019725	90	12 (13%)
Cell proliferation	0008283	1067	185 (17%)
Cell recognition	0008037	1820	284 (16%)
Cell-cell signalling	0007267	2582	377 (15%)
Chemi-mechanical coupling	0006943	102	15 (15%)
Co-enzyme and prosthetic group metabolism	0006731	1163	203 (17%)
Cytoskeleton organisation and biogenesis	0007010	1536	340 (22%)
DNA metabolism	0006259	1470	291 (20%)
Electron transport	0006118	3286	605 (18%)
Embryonic development	0009790	779	112 (14%)
Energy pathways	0006091	1497	279 (19%)
Growth	0040007	1405	243 (17%)
Host-pathogen interactions	0030383	4051	247 (14%)
Ion transport	0006811	2474	453 (18%)
Lipid metabolism	006629	859	182 (21%)
Mitochondria organisation and biogenesis	0007005	1273	246 (19%)
Morphogenesis	0009653	301	40 (14%)
Protein biosynthesis	0006412	1060	248 (23%)
Protein modification	0006412	307	79 (26%)
Protein transport	0015031	2031	516 (25%)
Regulation of gene expression; epigenetic	0040029	3969	685 (17%)
Reproduction	0000003	141	15 (11%)
Response to endogenous stimuli	0009719	103	26 (25%)
Response to stress	0006950	427	87 (20%)
Signal transduction	0007165	4158	620 (15%)
Transcription	0006350	3697	625 (17%)
Unknown biological processes	0000004	11034	1104 (10%)

The percentage of genes in each ontology group that shows differential expression between the short-term cell culture groups and normal controls is also shown in brackets.

K-means clustering was used to investigate the expression patterns of the 4870 gene probe sets between the 3 tumour culture Groups. Due to the large number of genes investigated, the k-means approach was used to cluster the genes into 20 sets.

Observations regarding the expression pattern for each set were then made (Table 5.8). Sets 1, 4 and 12 show similar gene expression patterns and could be grouped for further analysis, along with sets 9, 10 and 13; 5, 6 and 7; 14 and 17; and 3, 15, 16 and 18. Sets 2, 8, 11, 19 and 20 show specific expression patterns compared to any other set.

Table 5.8 K-means clustering of the 4870 differentially expressed genes between the 3 tumour Groups.

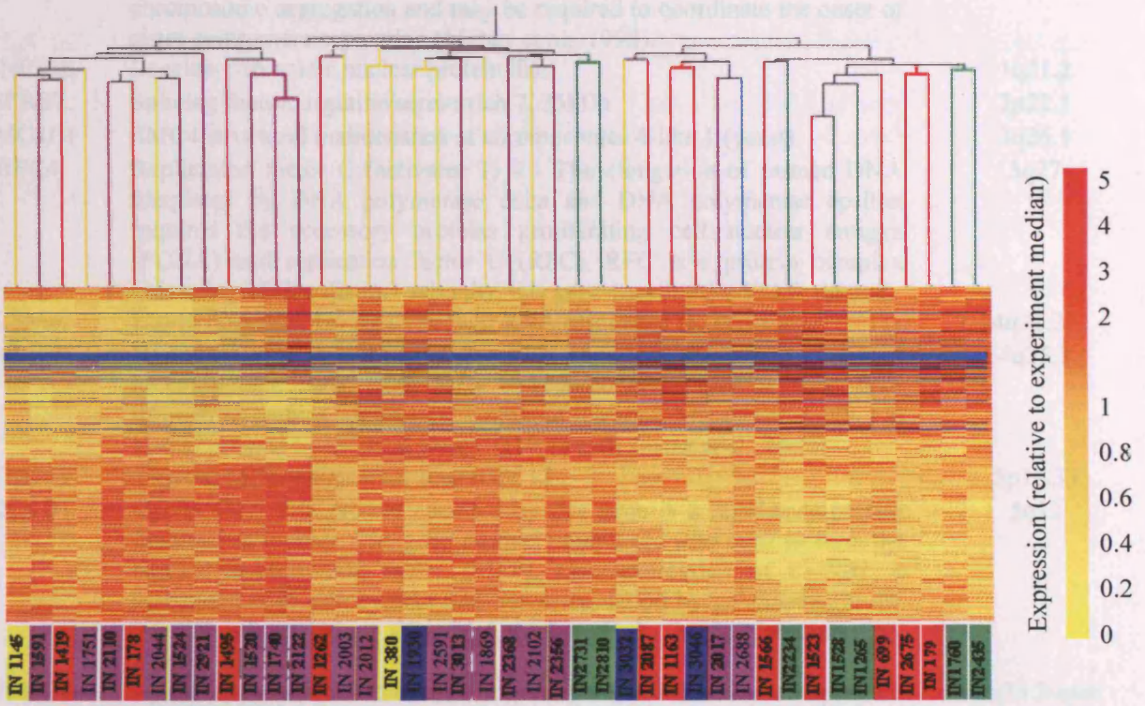
Set No.	No. of genes	Group 1	Group 2	Group 3
1	229	High	High	Low
2	308	Low	Low	High (sub group only)
3	299	High		Low
4	173	High	High	Low
5	399	Low	High	High
6	371	Low	High	High
7	250	Low	High	High
8	549			Low (sub group only)
9	329	High	Low	Low
10	366	High	Low	Low
11	144			High
12	162	High	High	Low
13	155	High	Low	Low
14	163	Low	High	Low
15	239	High		Low
16	130	High		Low
17	199	Low	High	Low
18	349	High		Low
19	89	Low		High
20	123	High	Low	

The Table indicates high or low gene expression in specific tumour Groups compared to the remaining Groups and normal controls.

In the dendrogram of Figure 5.6, the 308 genes from k-means cluster set 2 are highlighted blue. This set of genes was the only group to show high-expression in the second sub-group of Group 3 tumours. In total, 94 probe sets are tightly clustered by expression pattern (main blue band in Figure 5.6). In total, 53 probe sets correlated with the 4682 probe sets that characterise the paediatric short-term cell culture Groups. The remaining 41 probes, representing 38 genes, were only found in the 4870 probe set list that characterise the Groups when the adult short-term cell cultures are added. As this group of genes showed a unique expression pattern and was a reasonably small number,

the function of this set of genes was investigated by database mining to determine if a common biological function could be linked to the expression pattern (Table 5.9).

Figure 5.6 The dendrogram illustrates the clustering of the paediatric and adult short-term cell cultures and highlights the 308 genes from k-means cluster set 2 that show high-expression in the second sub-group of Group 3 tumours (genes highlighted blue). In total, 94 probe sets are tightly clustered by expression pattern.



The dendrogram colour saturation is proportional to the magnitude of the difference from the experiment median, ranging from red (high-expression) to yellow (low-expression). The tumour grades are according to the 2007 grading scheme; PAI=purple; DAII=yellow; paediatric GBMIV=red; adult GBMIV=green. The genes highlighted blue in the dendrogram are the 308 probe sets for set 2 of the k-means clustering approach. The tight blue band consists of 121 probe sets.

Table 5.9 The common names, functions and locations of the 38 genes that showed high-expression in the sub-group of Group 3 tumours compared to all other tumours.

Common Name	Description according to NCBI Locus Link	Chromosome Location
M96	Metal response element binding transcription factor 2	1p22.1
KIF2C	Kinesin family member 2C - Proteins of this family are microtubule-dependent molecular motors that transport organelles and move chromosomes during cell division. KIF2C is important for anaphase chromosome segregation and may be required to coordinate the onset of sister centromere separation (Maney et al., 1998).	1p34.1
ANP32E	Leucine-rich acidic nuclear protein like	1q21.2
SFRS7	Splicing factor, arginine/serine-rich 7, 35kDa	2p22.1
SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	3q26.1
RFC4	Replication factor C (activator 1) 4 - The elongation of primed DNA templates by DNA polymerase delta and DNA polymerase epsilon requires the accessory proteins proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). RFC is a protein complex consisting of five distinct subunits, this gene encodes the 37 kD subunit.	3q27
LSM6	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	4q31.21
KLIP1	Hypothetical protein FLJ23468 – This gene is expressed in nucleated hematopoietic cells, from early embryonic hematopoietic stem cells through to mature adult blood lymphoid lineages. Membrane KLIP-1 is also expressed by the fetal liver CD34(+) cells (Prost et al., 2002).	4q35.1
TRIP13	Thyroid hormone receptor interactor 13	5p15.33
CCNB1	Cyclin B1 - The protein encoded by this gene is a regulatory protein involved in mitosis. The gene product complexes with cdc2 to form the maturation-promoting factor (MPF). Over-expression of *CCNB1 is* associated with astrocytoma and down-regulation by geldanamycin has been shown to induce G2 arrest. (Allan et al., 2000; Nomura et al., 2007).	5q12
HMMR	Hyaluronan-mediated motility receptor (RHAMM) - The receptor for hyaluronan mediated motility has been reported to mediate migration, transformation, and metastasis. Increased *RHAMM* expression has been found in endometrial carcinomas (Rein et al., 2003) as well as other cancers. This protein can directly affect centrosomal structure and spindle integrity potentially modulating apoptosis and cell cycle progression (Maxwell et al., 2005).	5q33.2-qter
PTTG1	Pituitary tumor-transforming 1 – This protein prevents separins from promoting sister chromatid separation. It is an APC/C substrate that associates with a separin until activation of the APC/C. The gene product has transforming activity *in vitro* and tumorigenic activity *in vivo*. This gene is highly expressed in various tumours and over-expression in astrocytoma is a marker of poor prognosis (Genkai et al., 2006)	5q35.1
MCM3	MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) - The protein encoded by this gene is one of the highly conserved MCM proteins that are involved in genome replication. The protein complex formed by MCM proteins is a key component of the pre-replication complex and is involved in the formation of replication forks and recruitment of other DNA replication proteins. Over-expression of MCM3 has been linked to reduced survival in DAII, AAIII and GBMIV (Soling et al., 2005).	6p12
NUP153	Nucleoporin 153kDa	6p22.3
CBX3	Chromobox homolog 3 (HP1 gamma homolog, Drosophila) - The protein encoded for by this gene binds DNA and is a component of heterochromatin.	7p15.2
PFTK1	PFTAIRE protein kinase 1	7q21-q22

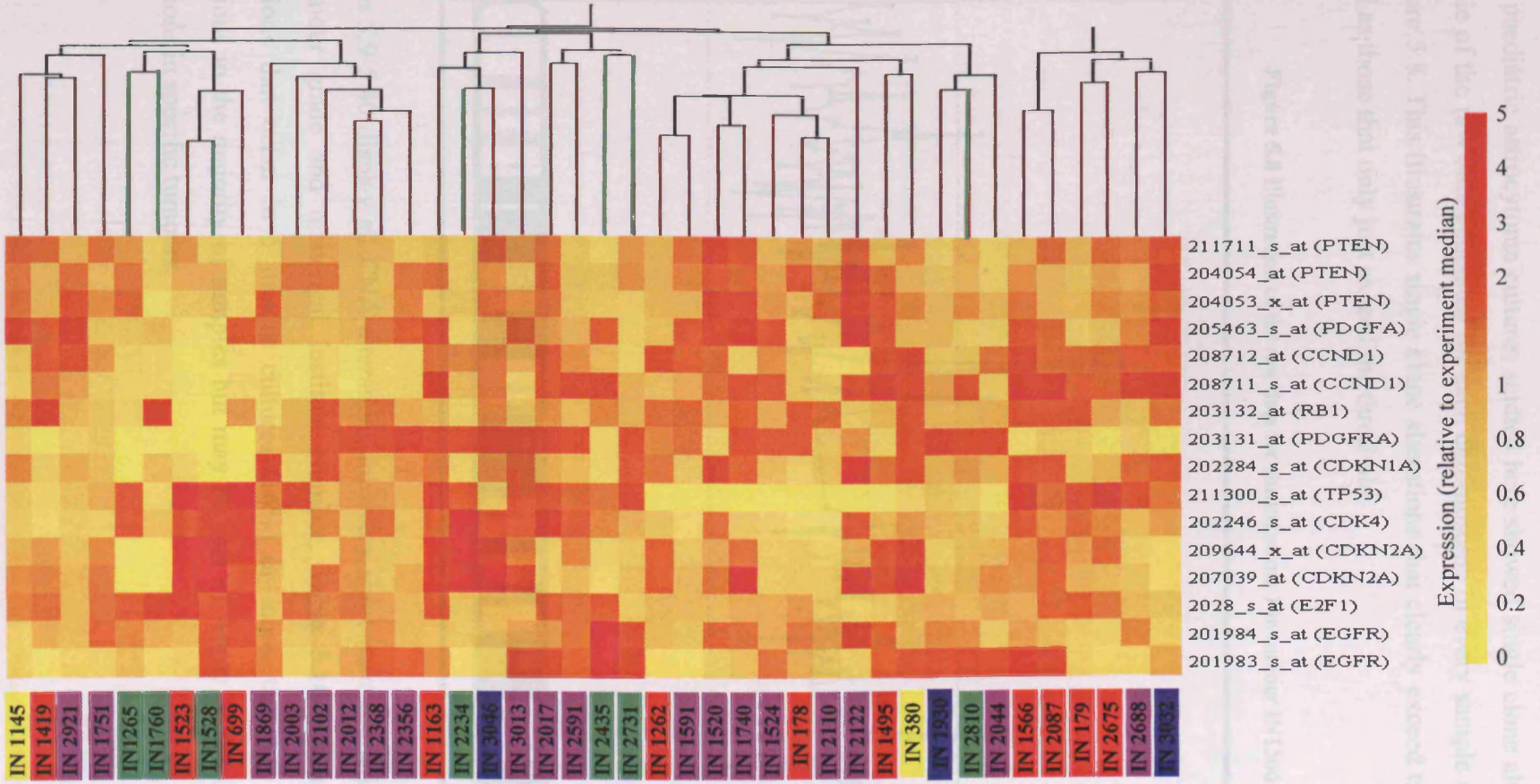
MTB	Hypothetical protein FLJ20311	7q36.3
MELK	Maternal embryonic leucine zipper kinase - *MELK* over-expression is thought to affect mammary carcinogenesis through the inhibition of the pro-apoptotic function of Bcl-GL (Lin et al., 2007).	9p13.1
MKI67	Antigen identified by monoclonal antibody Ki-67 - A gradual increase in Ki-67 antigen expression was observed in agreement with mitotic index and histopathological classification of astrocytoma (Faria et al., 2006).	10q25-qter
CNAP1	Chromosome condensation-related SMC-associated protein 1	12p13.3
RACGAP1	Rac GTPase activating protein 1 – This gene was found to be over-expressed in epithelial ovarian cancer (Lu et al., 2004).	12q13.12
RFC5	Replication factor C (activator 1) 5 – Refer to RFC4; this gene encodes the 36.5kDa subunit.	12q24.2-q24.3
CKAP2	Cytoskeleton associated protein 2 – The protein encoded by this gene is linked to the microtubule network. *CKAP2* was found to be over-expressed in gastric adenocarcinomas (Bae et al., 2003).	13q14
NUSAP1	Nucleolar protein ANKT – The protein of this gene localises prominently to central spindle microtubules during mitosis. Gene expression is selective to proliferating cells (Raemaekers et al., 2003).	15q14
BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast) - This gene encodes a kinase involved in spindle checkpoint function. The protein has been localized to the kinetochore and plays a role in the inhibition of APC/C, delaying the onset of anaphase and ensuring proper chromosome segregation. Impaired spindle checkpoint function has been found in many forms of cancer, the over expression *BUB1B* is associated with chromosomal instability in bladder cancer (Yamamoto et al., 2007).	15q15
CCNB2	Cyclin B2 - Cyclin B2 is a member of the B-type cyclins. Cyclins B1 and B2 associate with cdc2 and are essential components of the cell cycle regulatory machinery. Cyclin B1 co-localizes with microtubules, whereas cyclin B2 is primarily associated with the Golgi region. Cyclin B2 also binds to transforming growth factor beta RII and thus cyclin B2/cdc2 may play a key role in transforming growth factor beta-mediated cell cycle control. Over-expression of Cyclin B2 was found in colorectal adenocarcinomas (Park et al., 2007).	15q21.2
AURKB	Serine/threonine kinase 12 - Chromosomal segregation during mitosis is regulated by kinases and phosphatases. Aurora kinase B (AURKB) localizes to microtubules near kinetochores with over-expression of this gene inducing multicellularity and polyploidy. Over-expression of *AURKB* was correlated with a shortened survival in glioblastoma (Zeng et al., 2007).	17p13.1
FLJ10156	Hypothetical protein FLJ10156	17p13.2
FLJ10534	Hypothetical protein FLJ10534	17p13.3
CDC6	CDC6 cell division cycle 6 homolog (S. cerevisiae) - This protein functions as a regulator in the early steps of DNA replication. It localizes to the cell nucleus during the G1 phase of the cell cycle, but translocates to the cytoplasm at the start of S phase. The subcellular translocation of this protein during the cell cycle is regulated through its phosphorylation by Cdks. Transcription of this protein was reported to be regulated in response to mitogenic signals via transcriptional control mechanisms involving E2F proteins. Moreover, activation of TP53 by DNA damage results in enhanced Cdc6 destruction by the APC/C complex (Duursma and Agami, 2005).	17q21.3
JUNB	Ribonuclease H2, large subunit. JUNB is a proto-oncogene that functions as a regulator of erythroid cell survival, proliferation, and differentiation. It is one of many DNA binding proteins that form AP-1 dimers. Amongst the Jun proteins that are involved in this dimerisation, c-Jun positively regulates cell proliferation through the repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription. JunB antagonises these actions, by up-regulating tumor	19p13.12

	suppressor genes and repressing cyclin D1 (Shaulian and Karin, 2001b).	
ASF1B	Anti-silencing function 1B. This gene encodes a member of the H3/H4 family of histone chaperone proteins. The encoded protein is the substrate of the tousled-like kinase family of cell cycle-regulated kinases, and may play a key role in modulating the nucleosome structure of chromatin by ensuring a constant supply of histones at sites of nucleosome assembly.	19p13.12
KIAA0186	KIAA0186 gene product	20p11.1
TOP1	Topoisomerase (DNA) I – The protein of this gene is an enzyme that adjusts the number of supercoils in DNA and altering topologic states during transcription Both type I and type II topoisomerases have been identified as clinically important targets for cancer chemotherapy and their inhibitors are central components in many therapeutic regimes (Giles and Sharma, 2005).	20q12-q13.1
UBE2C	Ubiquitin-conjugating enzyme E2C. This gene encodes a member of the E2 ubiquitin-conjugating enzyme family and is required for the destruction of mitotic cyclins and for cell cycle progression. Hepatocellular carcinomas with over-expression of *UBE2C* showed higher frequencies of tumor invasion and tumor de-differentiation. Furthermore, patients with tumours that showed high *UBE2C* expression also showed significantly worse disease-free survival rates compared to those with low *UBE2C* expression (Ieta et al., 2007).	20q13.11
C21orf45	Chromosome 21 open reading frame 45.	21q22.11
KIF4A	Kinesin family member 4A. Kinesins, such as KIF4A, are microtubule-based motor proteins that generate directional movement along microtubules. They are involved in many crucial cellular processes, including cell division.	Xq13.1
HMGB3	High-mobility group box 3. HMGB3 is a member of a family of chromatin-binding proteins that can alter DNA structure to facilitate transcription factor binding.	Xq28

To investigate the expression pattern of those genes commonly differentially expressed in adult astrocytoma, as discussed in the introduction, in the paediatric astrocytoma of this study, the expression profile of specific genes including *TP53*, *PDFRA*, *PDGFA*, *RB*, *PTEN*, *EGFR*, *CDKN2A*, *CDKN1A*, *CCND1*, *CDK4* and *E2F1* were investigated in all short-term cell culture samples. No reliable expression data was available for *CDK6* or *MDM2*.

Figure 5.7 illustrates a dendrogram comparing the expression profile similarity of the short-term cell cultures from adult and paediatric tumours and 16 probe pairs representing the 11 genes previously described. According to the expression of these 11 genes, the samples do not cluster by Group or grade and the adult short-term cell cultures integrate with the paediatric short-term cell cultures. However, 4 sample cluster groups can be seen.

Figure 5.7 The dendrogram illustrates the expression pattern of 11 genes commonly differentially expressed in adult astrocytoma, in the short-term cell cultures of this study.

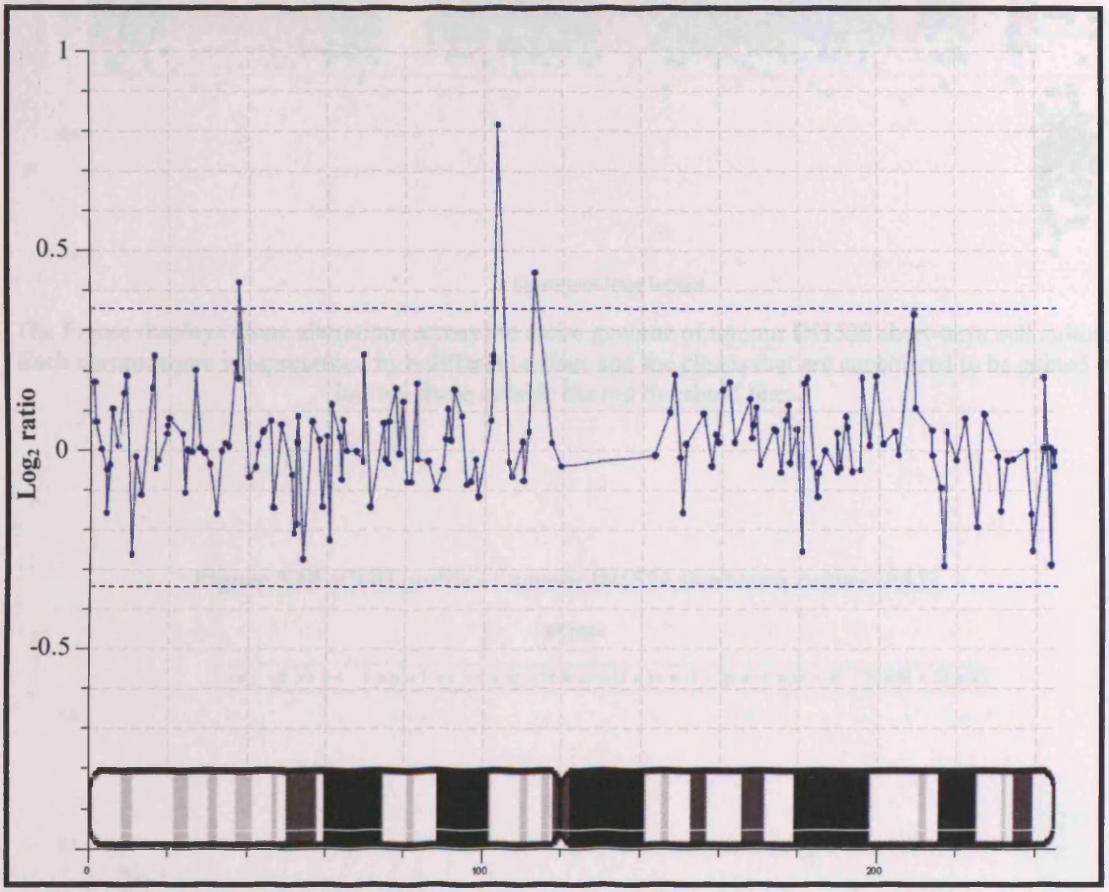


The branches of the dendrogram represent each sample, green branches are adult GBMIV short-term cell cultures and black branches are paediatric short-term cell cultures. The tumour grades shown correspond to the 2007 WHO grading scheme; PAI = purple; DAII = yellow; AAIII = dark blue; paediatric GBMIV = red; adult GBMIV = green. The dendrogram colour ranges from red (high-expression) to yellow (low-expression).

Genomic CNAs in paediatric astrocytoma short-term cell cultures

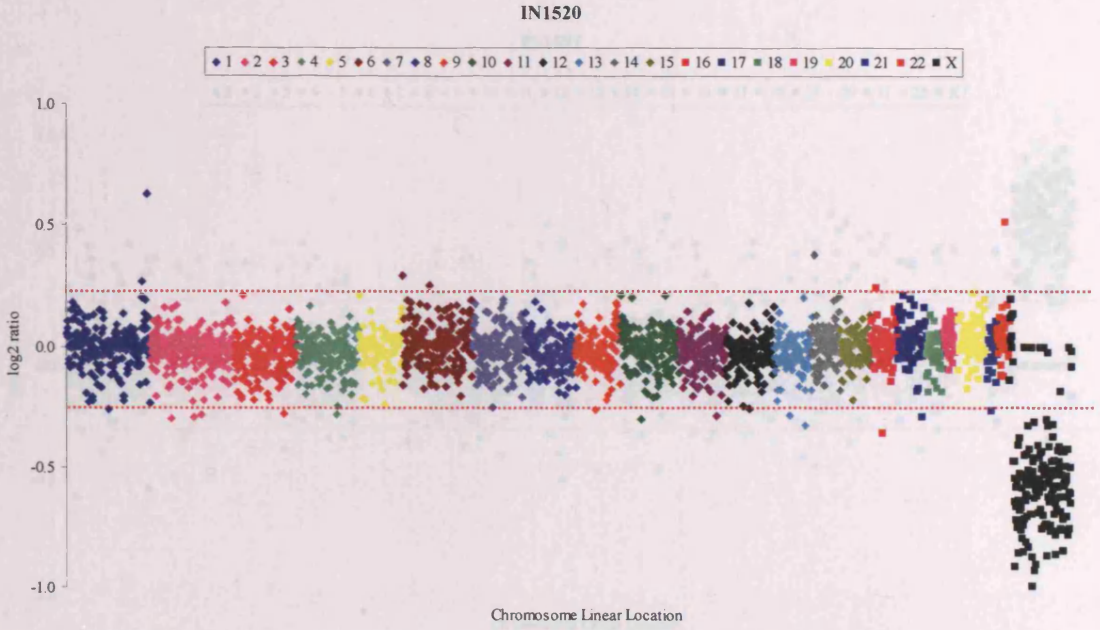
All of paediatric astrocytoma cultures studied here showed single clone alterations. An example of the raw data generated for each chromosome in every sample can be found in Figure 5.8. This illustrates single clone alterations that clearly exceed the threshold, as well as those that only just exceed the threshold.

Figure 5.8 Illustration of the raw data for chromosome 1 in tumour IN1566.



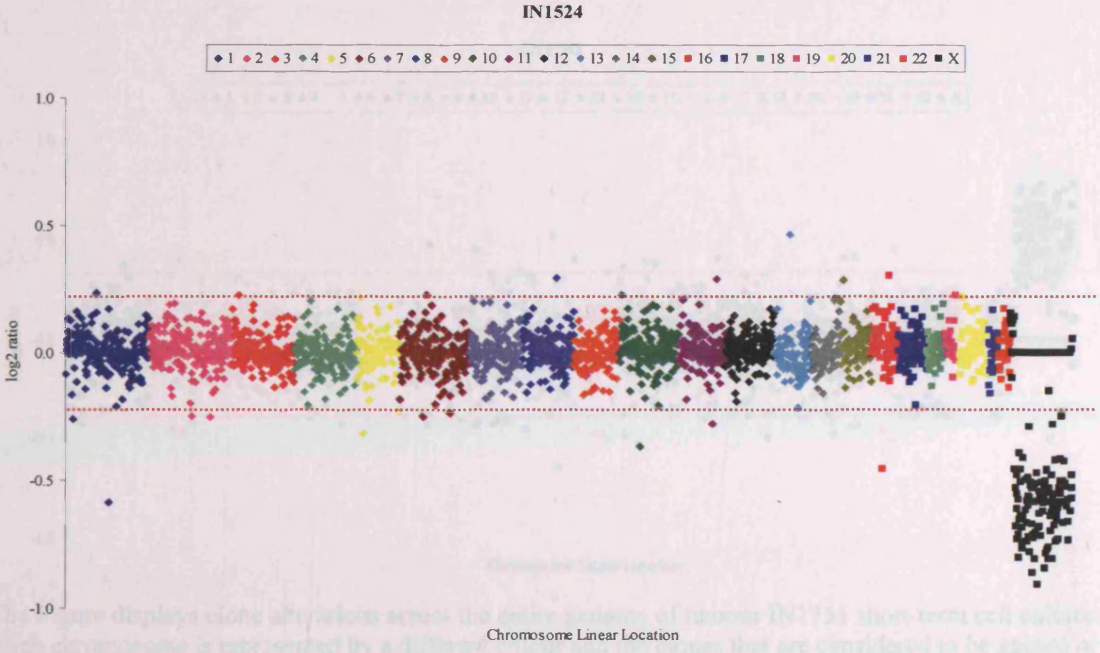
Figures 5.9-5.40 display all CNA alterations for every short-term cell culture, ordered by tumour grade and numerical Institute Number. Table 5.10 displays 37 clone alterations that occur in 5 tumour cultures (16%) or more, that are single clone alterations in the majority of samples but may be part of whole chromosome or arm alterations in specific tumours.

Figure 5.9 aCGH profile of tumour IN1520 short-term culture (PAI).



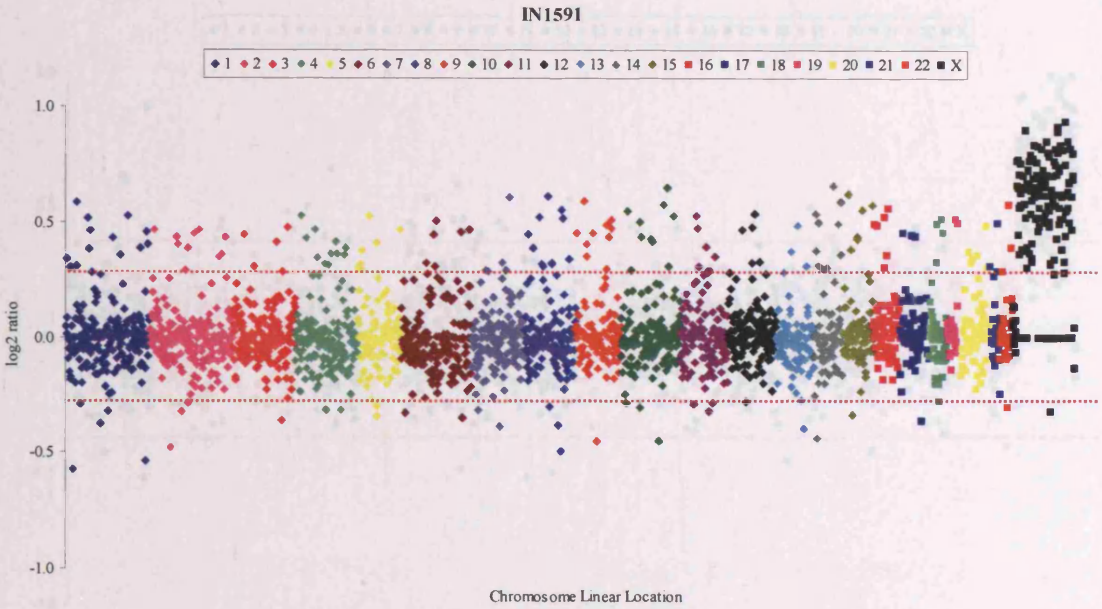
The Figure displays clone alterations across the entire genome of tumour IN1520 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.10 aCGH profile of tumour IN1524 short-term culture (PAI).



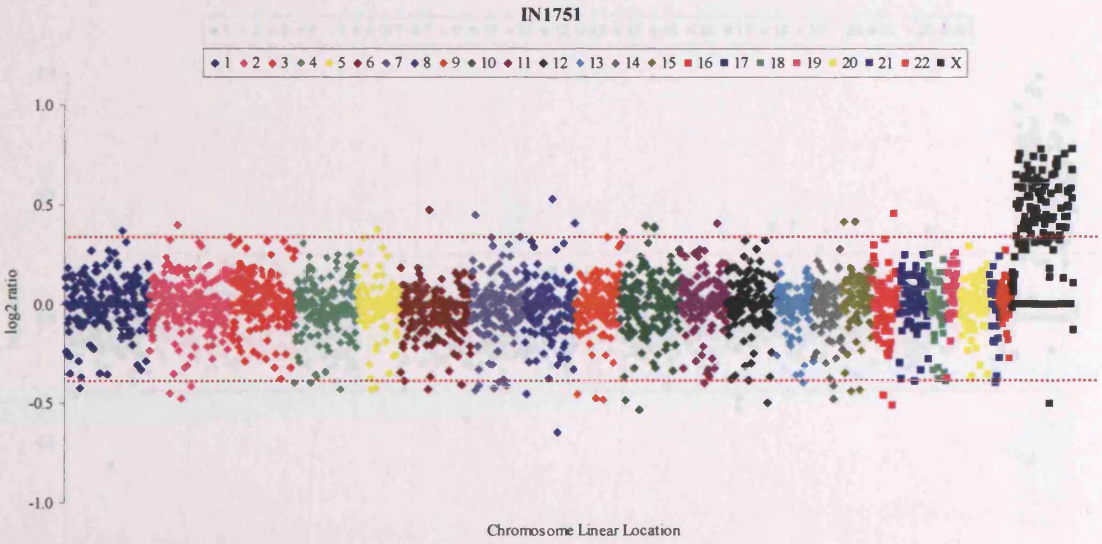
The Figure displays clone alterations across the entire genome of tumour IN1524 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.11 aCGH profile of tumour IN1591 short-term culture (PAI).



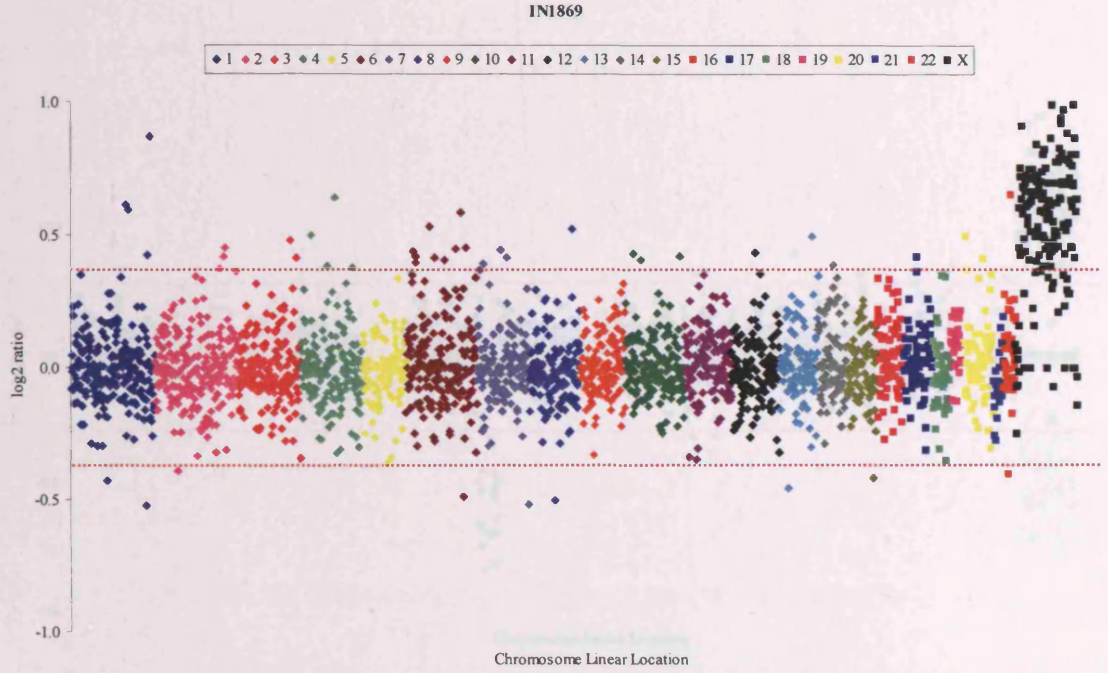
The Figure displays clone alterations across the entire genome of tumour IN1591short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.12 aCGH profile of tumour IN1751 short-term culture (PAI).



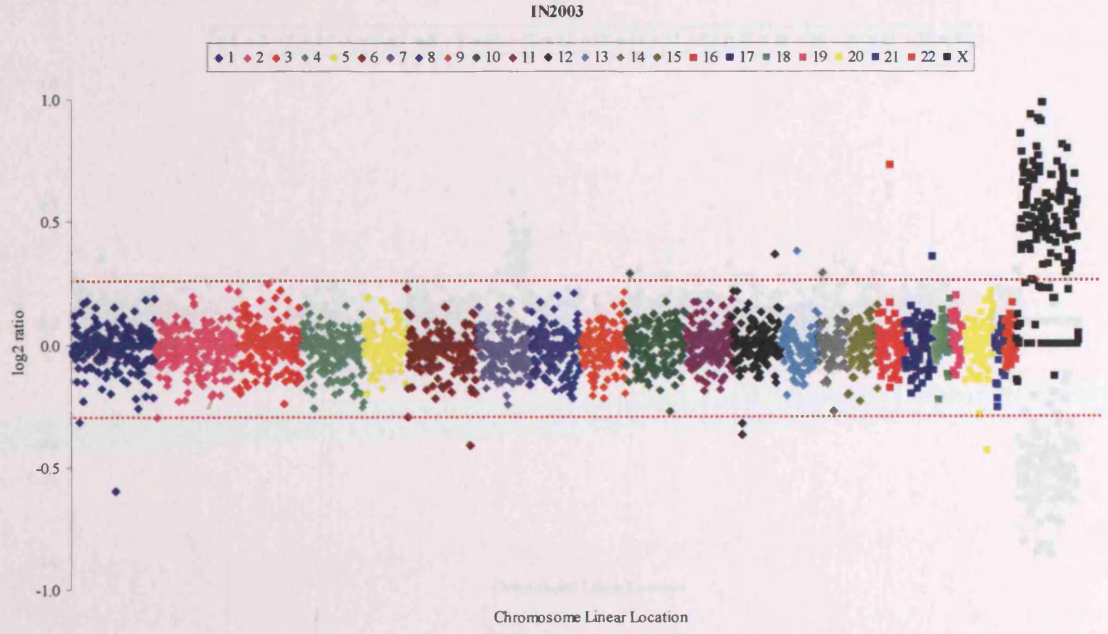
The Figure displays clone alterations across the entire genome of tumour IN1751 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.13 aCGH profile of tumour IN1869 short-term culture (PAI).



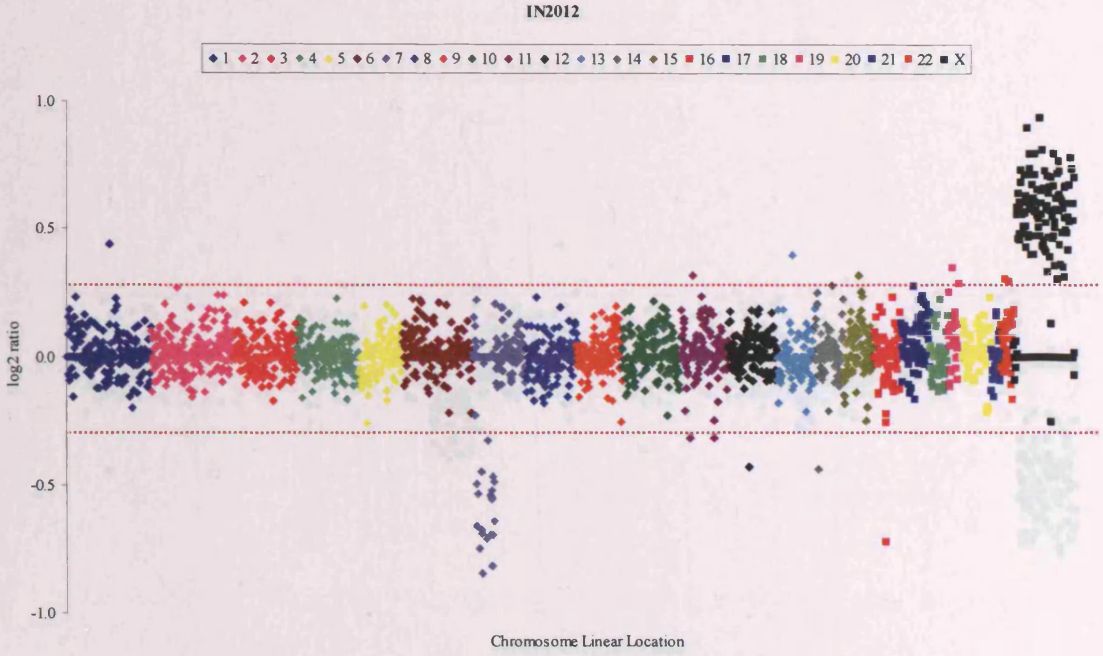
The Figure displays clone alterations across the entire genome of tumour IN1869short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.14 aCGH profile of tumour IN2003 short-term culture (PAI).



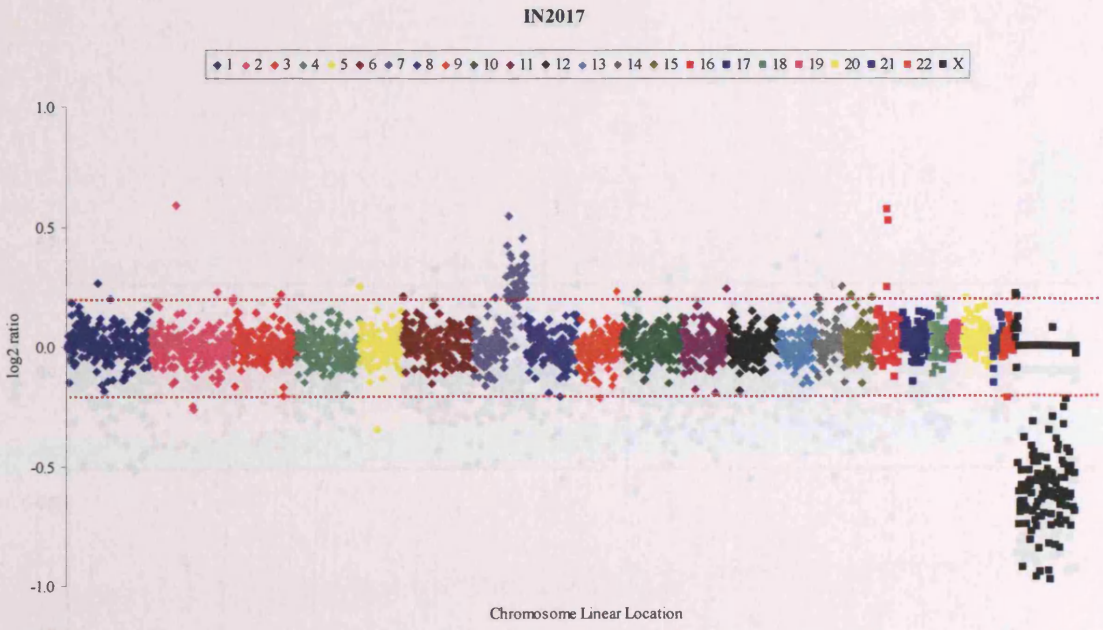
The Figure displays clone alterations across the entire genome of tumour IN2003 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.15 aCGH profile of tumour IN2012 short-term culture (PAI).



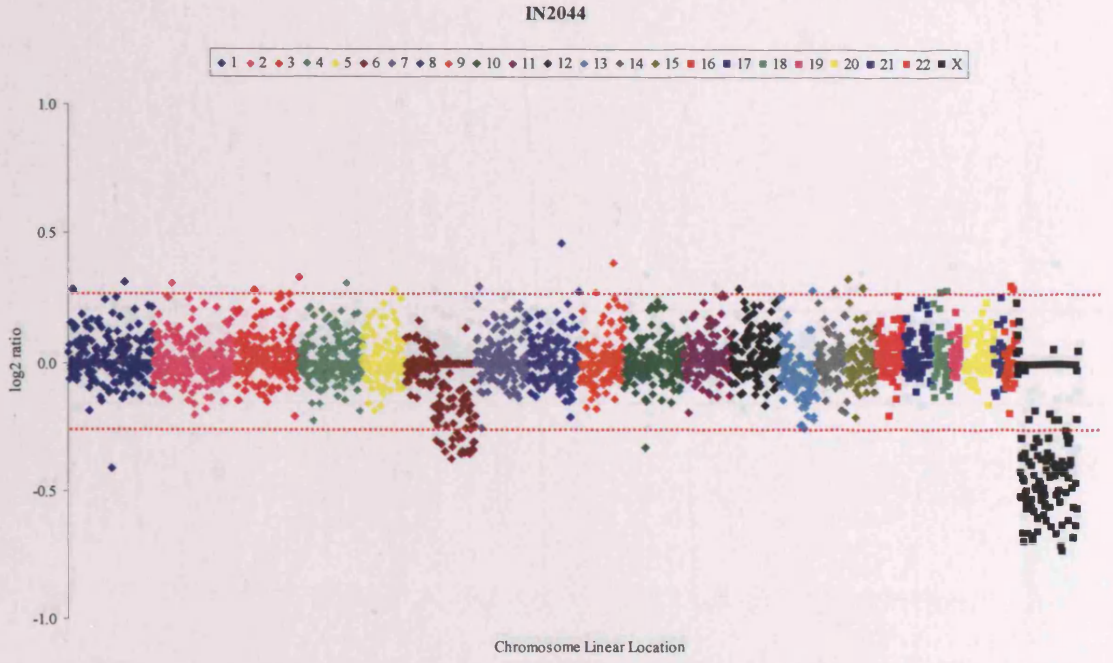
The Figure displays clone alterations across the entire genome of tumour IN2012 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.16 aCGH profile of tumour IN2017 short-term culture (PAI).



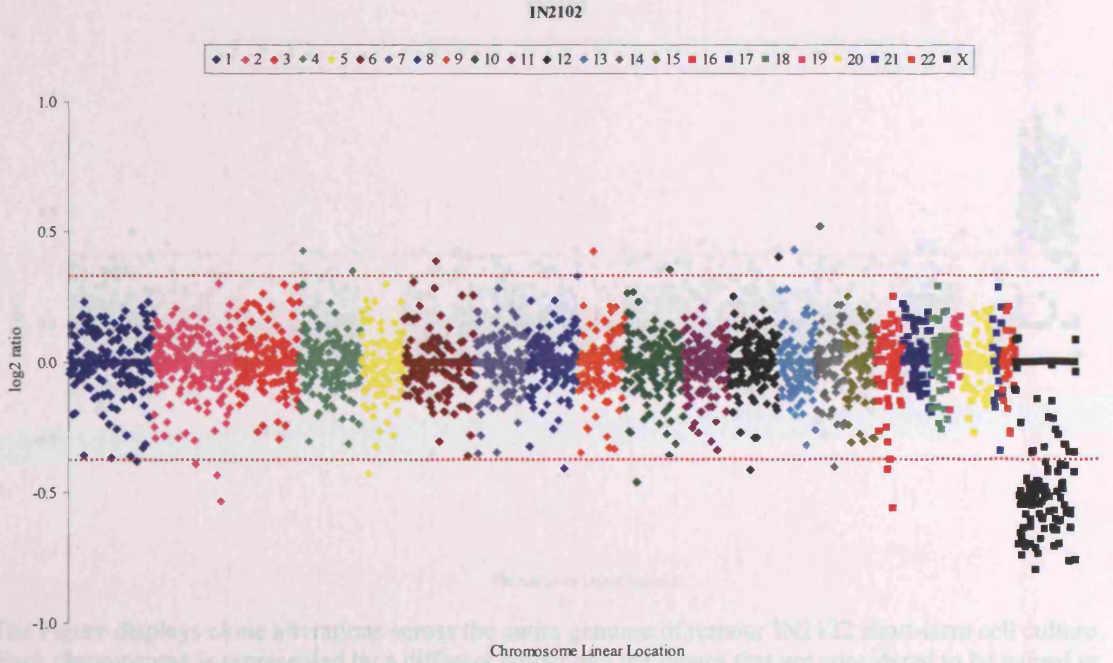
The Figure displays clone alterations across the entire genome of tumour IN2017 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.17 aCGH profile of tumour IN2044 short-term culture (PAI).



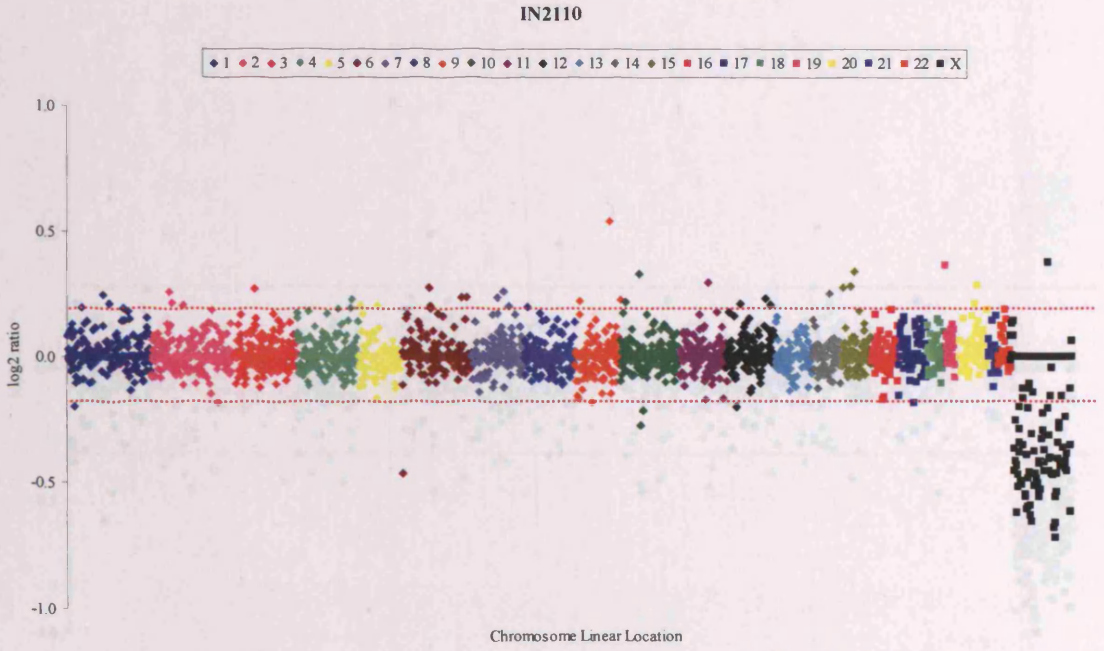
The Figure displays clone alterations across the entire genome of tumour IN2044 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.18 aCGH profile of tumour IN2102 short-term culture (PAI).



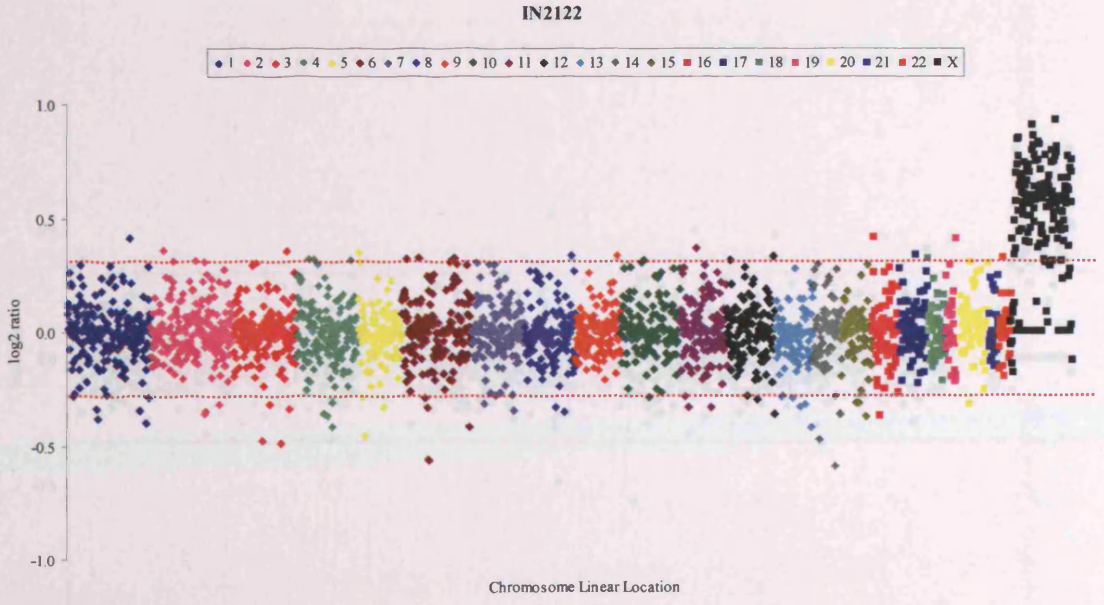
The Figure displays clone alterations across the entire genome of tumour IN2102 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.19 aCGH profile of tumour IN2110 short-term culture (PAI).



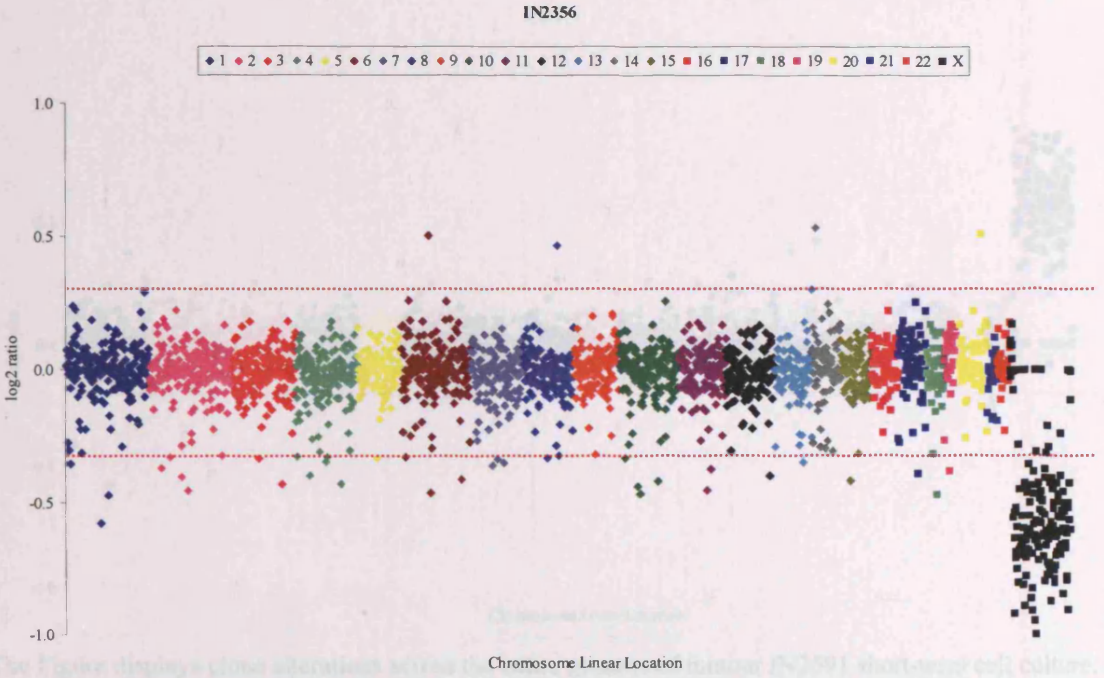
The Figure displays clone alterations across the entire genome of tumour IN2110short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.20 aCGH profile of tumour IN2122 short-term culture (PAI).



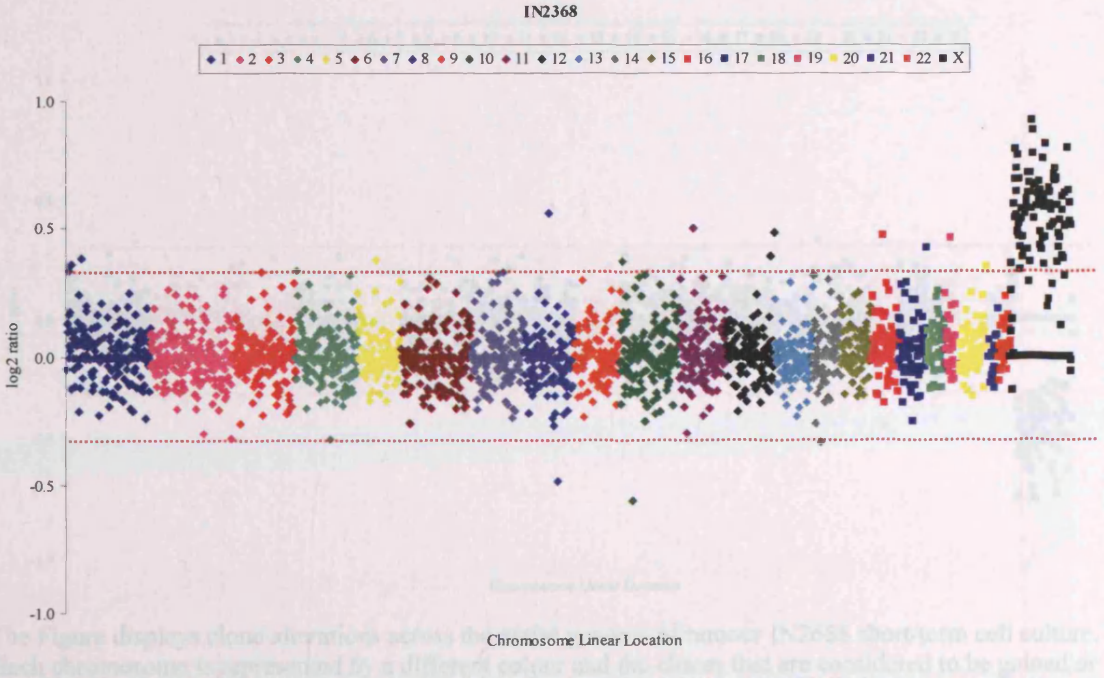
The Figure displays clone alterations across the entire genome of tumour IN2122 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.21 aCGH profile of tumour IN2356 short-term culture (PAI).



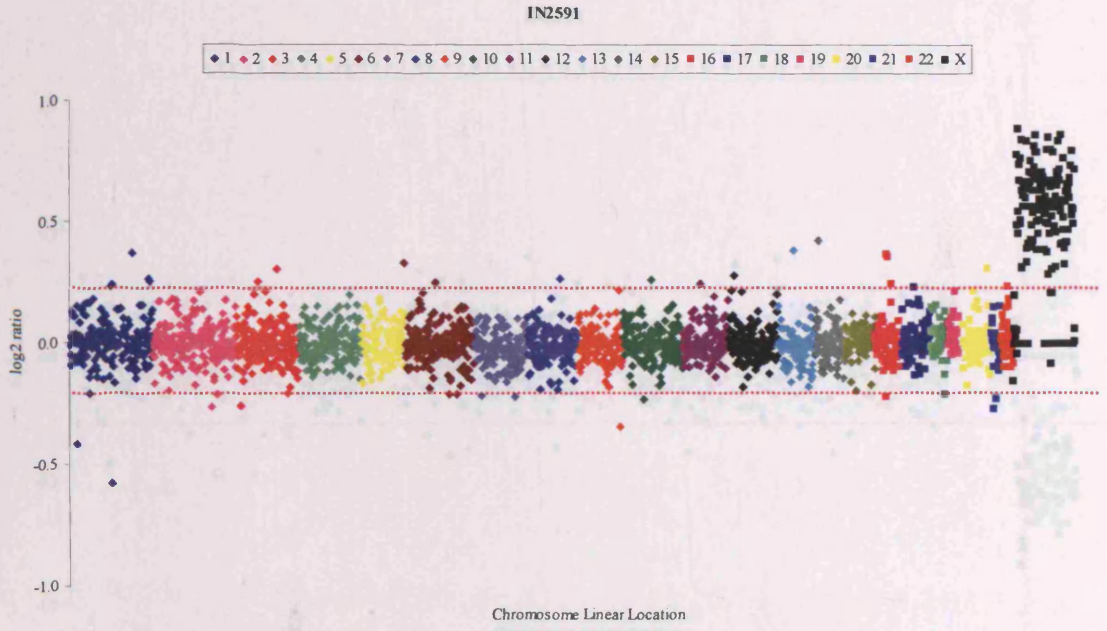
The Figure displays clone alterations across the entire genome of tumour IN2356 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.22 aCGH profile of tumour IN2368 short-term culture (PAI).



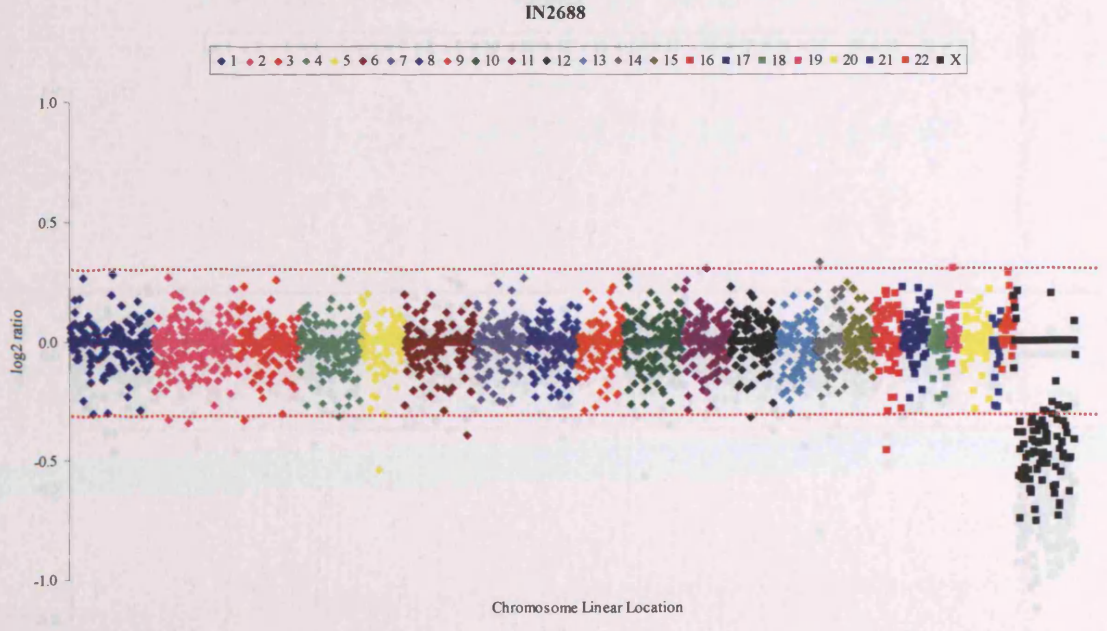
The Figure displays clone alterations across the entire genome of tumour IN2368 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.23 aCGH profile of tumour IN2591 short-term culture (PAI).



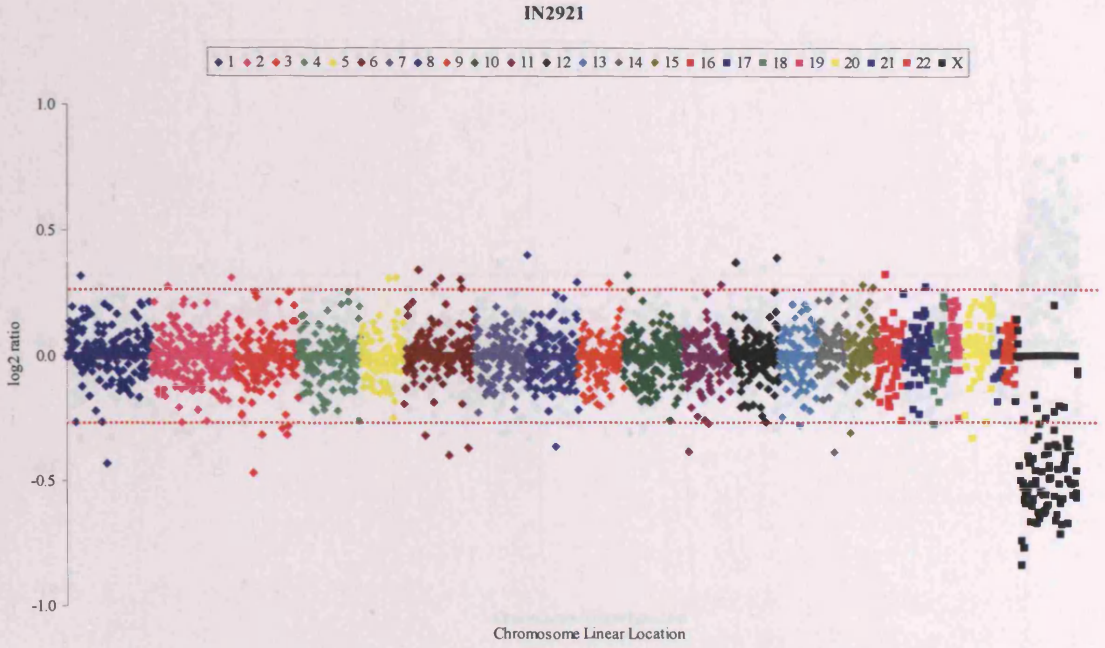
The Figure displays clone alterations across the entire genome of tumour IN2591 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.24 aCGH profile of tumour IN2688 short-term culture (PAI).



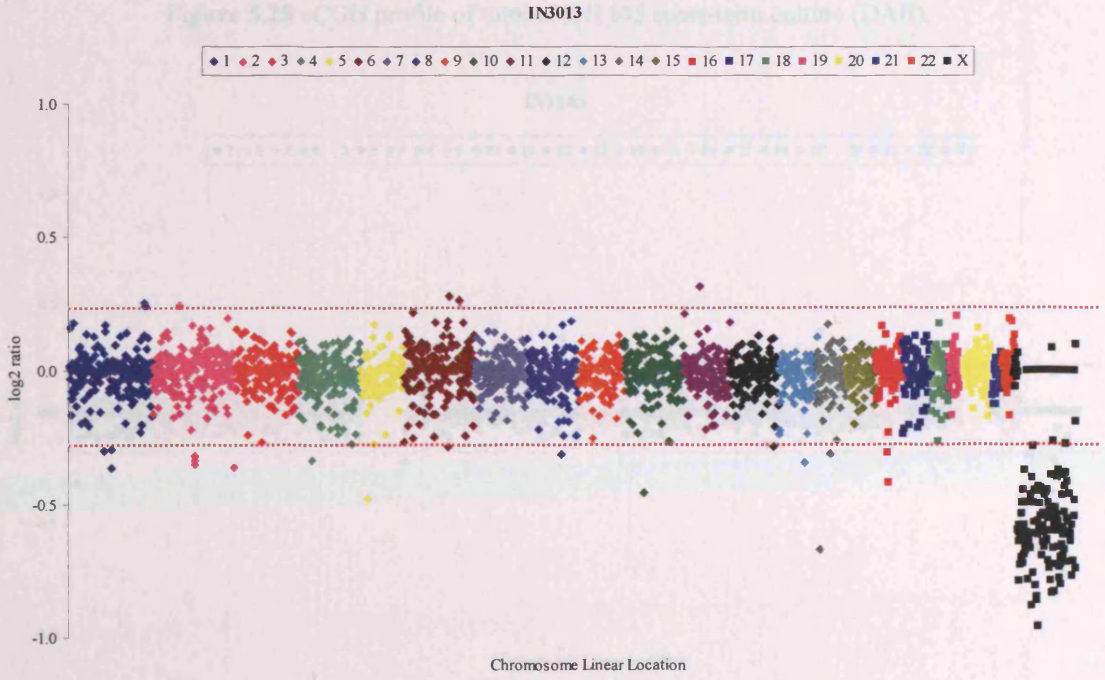
The Figure displays clone alterations across the entire genome of tumour IN2688 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.25 aCGH profile of tumour IN2921 short-term culture (PAI).



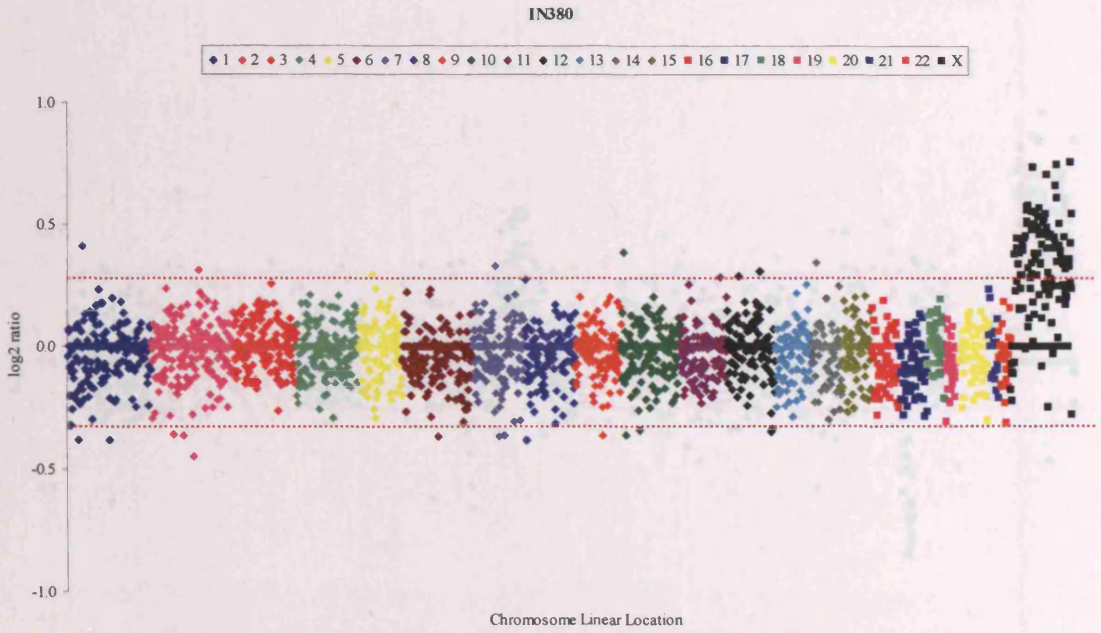
The Figure displays clone alterations across the entire genome of tumour IN2921 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.26 aCGH profile of tumour IN3013 short-term culture (PAI).



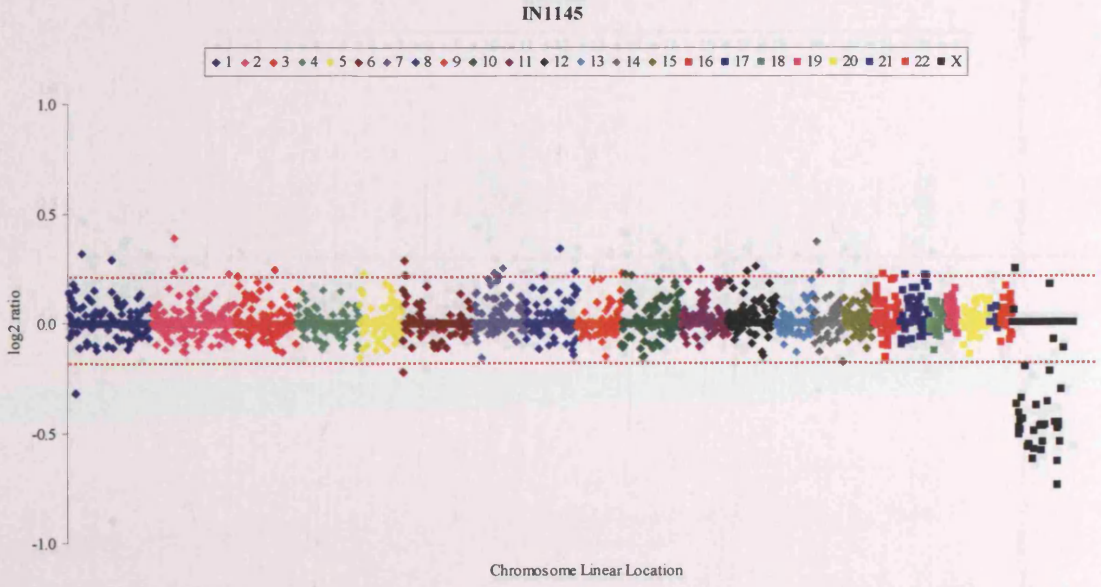
The Figure displays clone alterations across the entire genome of tumour IN3013 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.27 aCGH profile of tumour IN380 short-term culture (DAII).



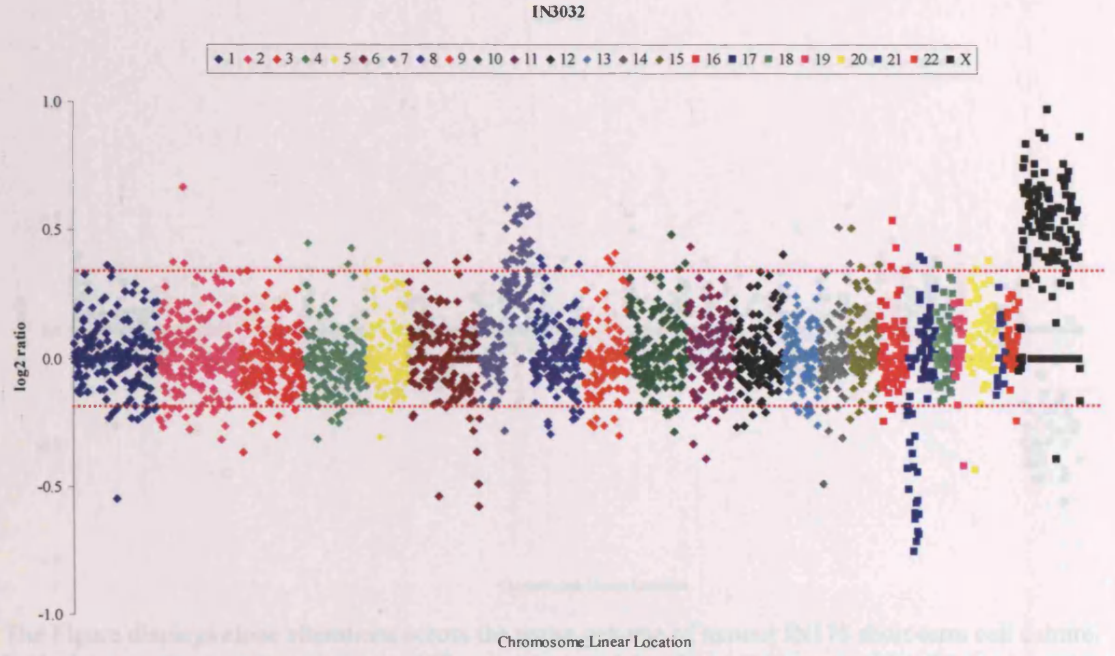
The Figure displays clone alterations across the entire genome of tumour IN380 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.28 aCGH profile of tumour IN1145 short-term culture (DAII).



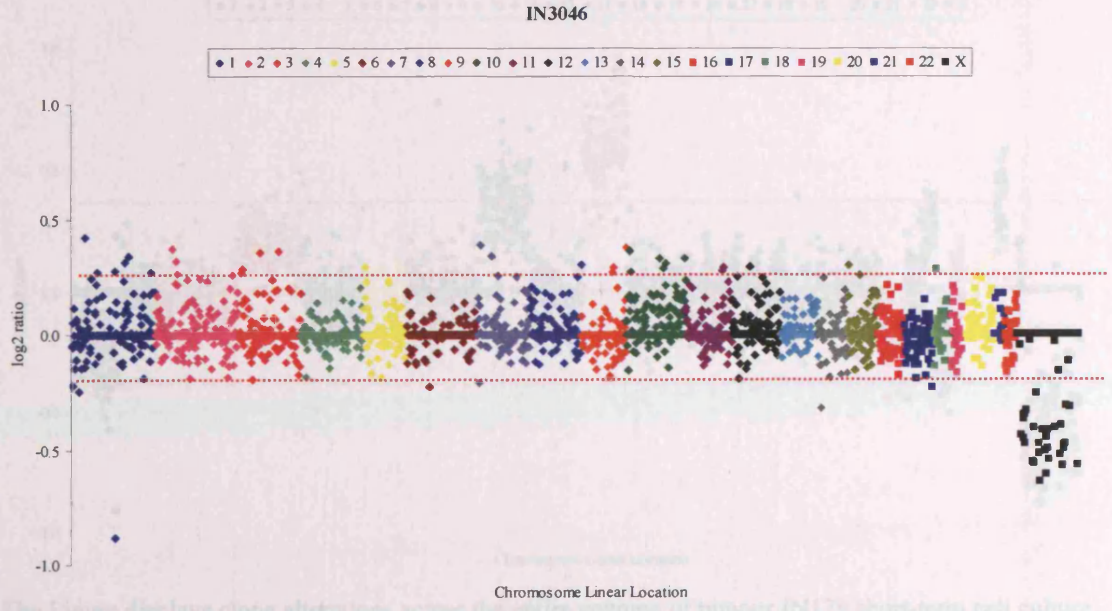
The Figure displays clone alterations across the entire genome of tumour IN1145short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.29 aCGH profile of tumour IN3032 short-term culture (AAIII).



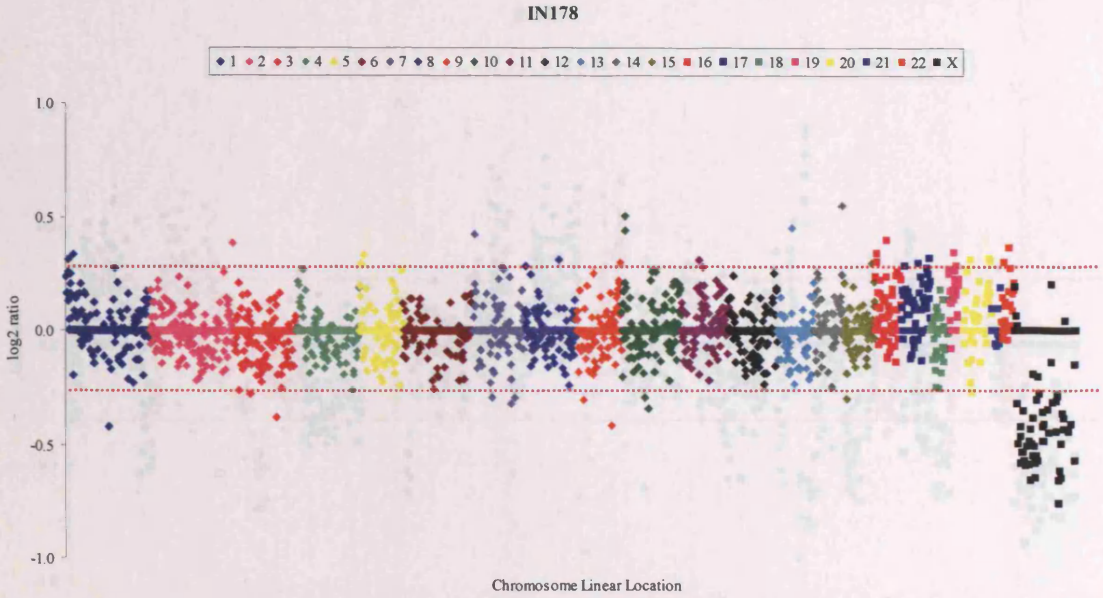
The Figure displays clone alterations across the entire genome of tumour IN3032 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.30 aCGH profile of tumour IN3046 short-term culture (AAIII).



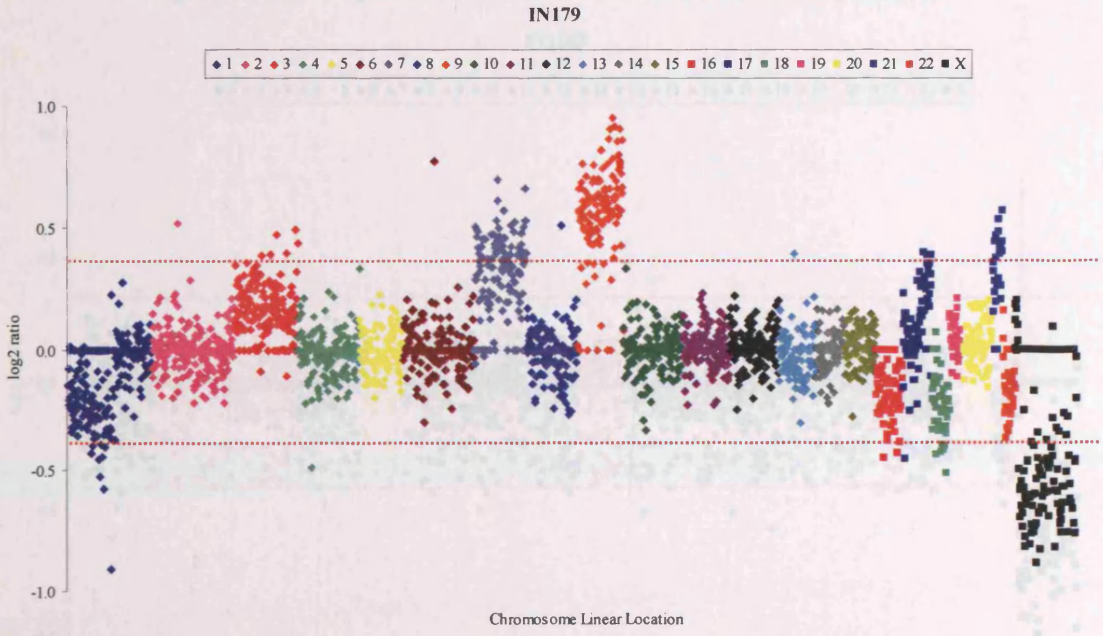
The Figure displays clone alterations across the entire genome of tumour IN3046 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.31 aCGH profile of tumour IN178 short-term culture (GBMIV).



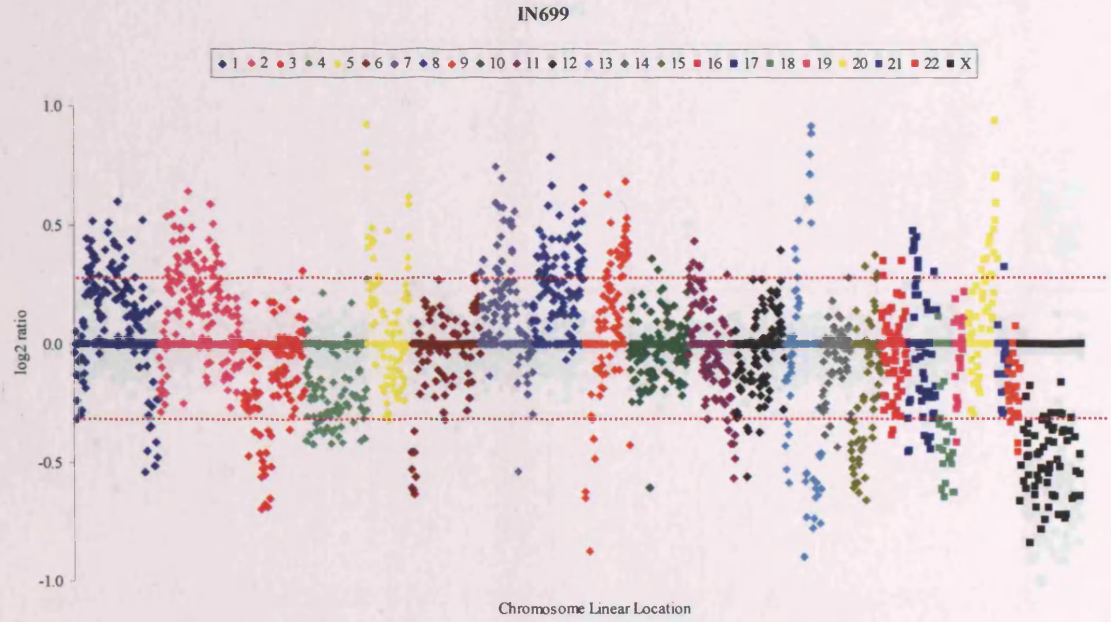
The Figure displays clone alterations across the entire genome of tumour IN178 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.32 aCGH profile of tumour IN179 short-term culture (GBMIV).



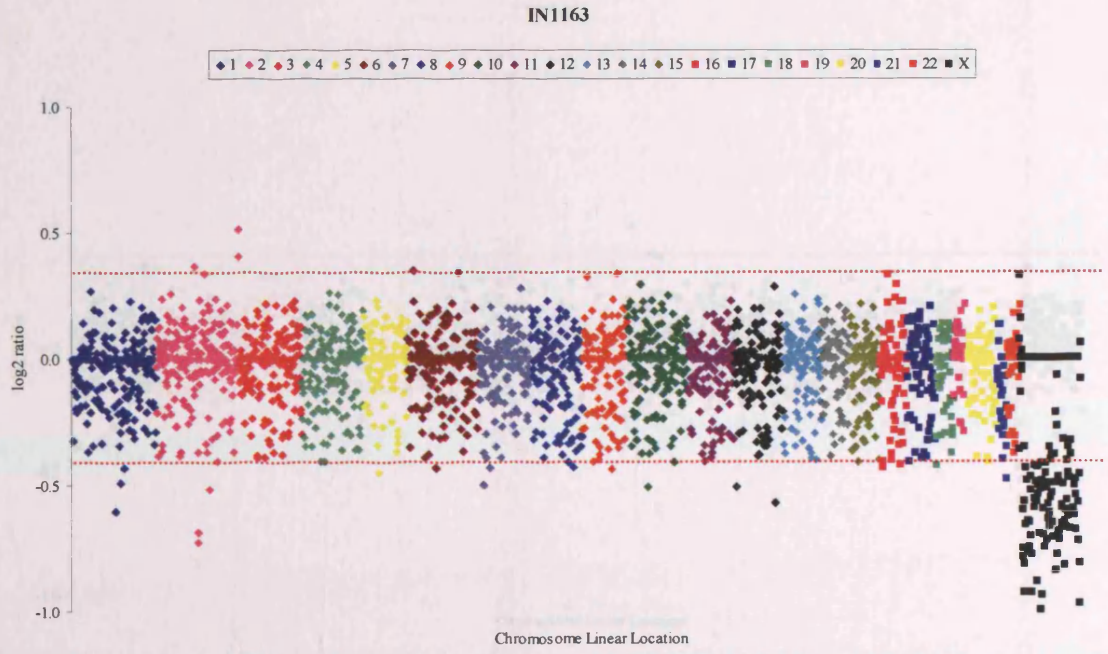
The Figure displays clone alterations across the entire genome of tumour IN179 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.33 aCGH profile of tumour IN699 short-term culture (GBMIV).



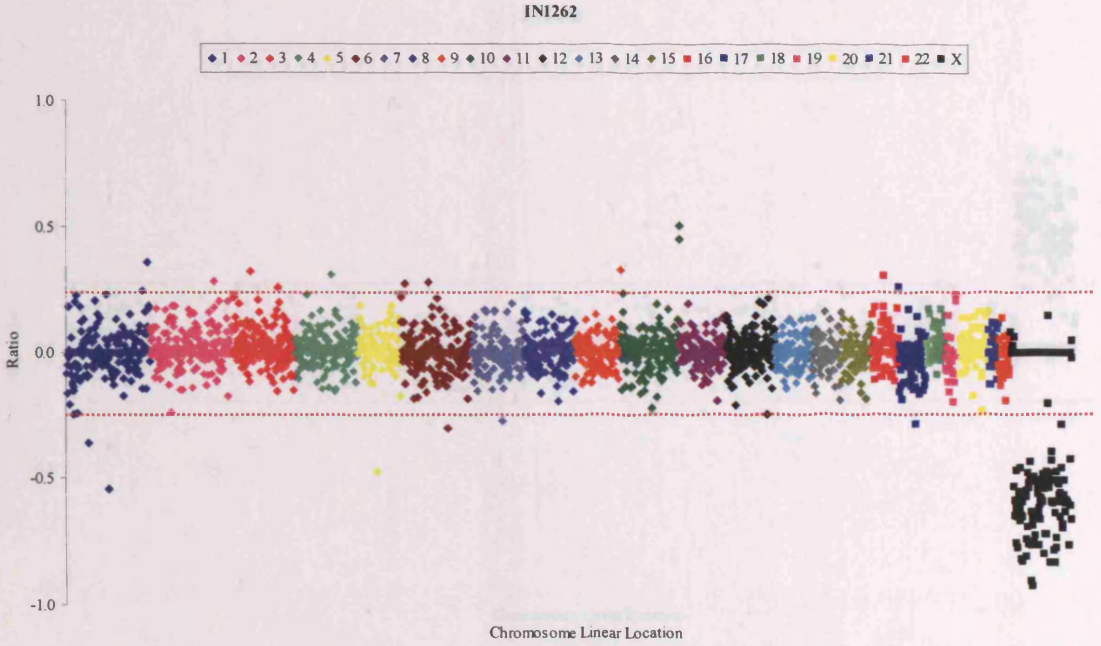
The Figure displays clone alterations across the entire genome of tumour IN699 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.34 aCGH profile of tumour IN1163 short-term culture (GBMIV).



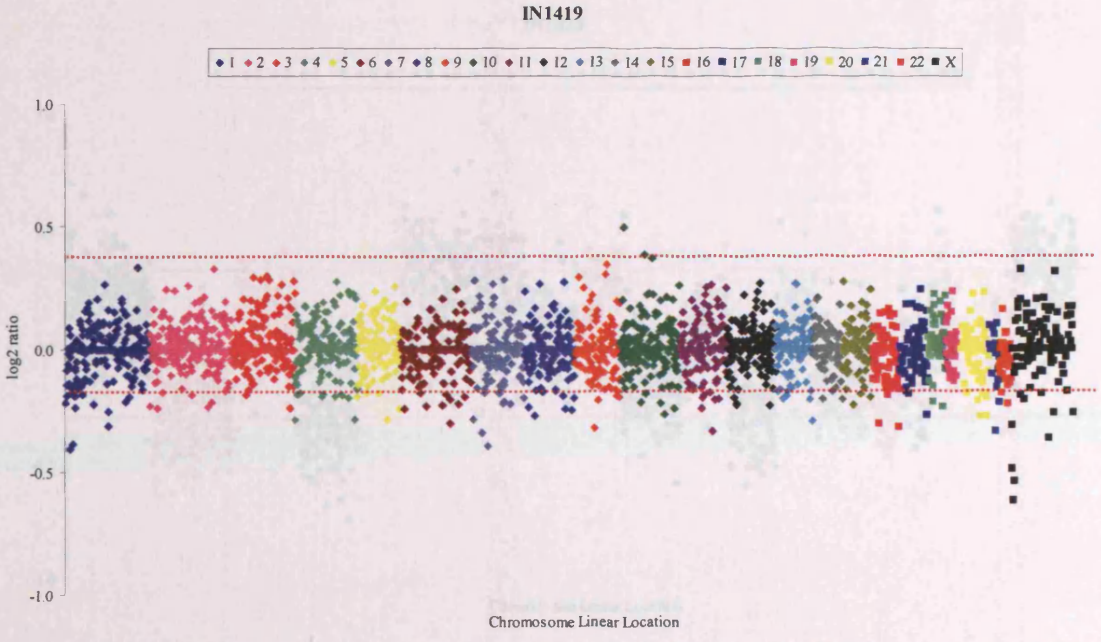
The Figure displays clone alterations across the entire genome of tumour IN1163 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.35 aCGH profile of tumour IN1262 short-term culture (GBMIV).



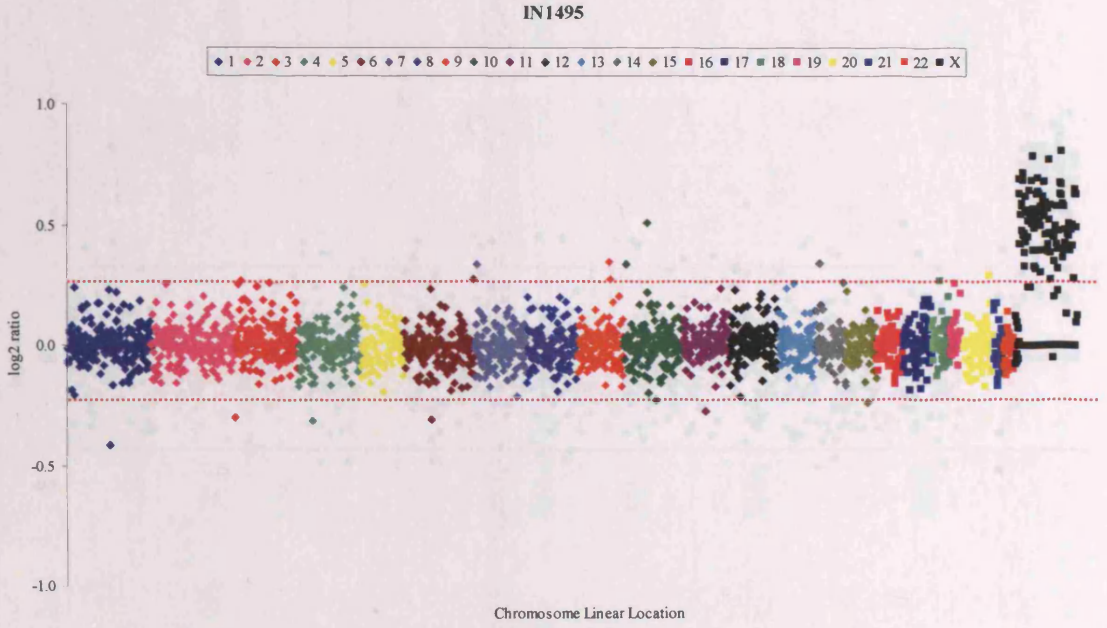
The Figure displays clone alterations across the entire genome of tumour IN1262 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.36 aCGH profile of tumour IN1419 short-term culture (GBMIV).



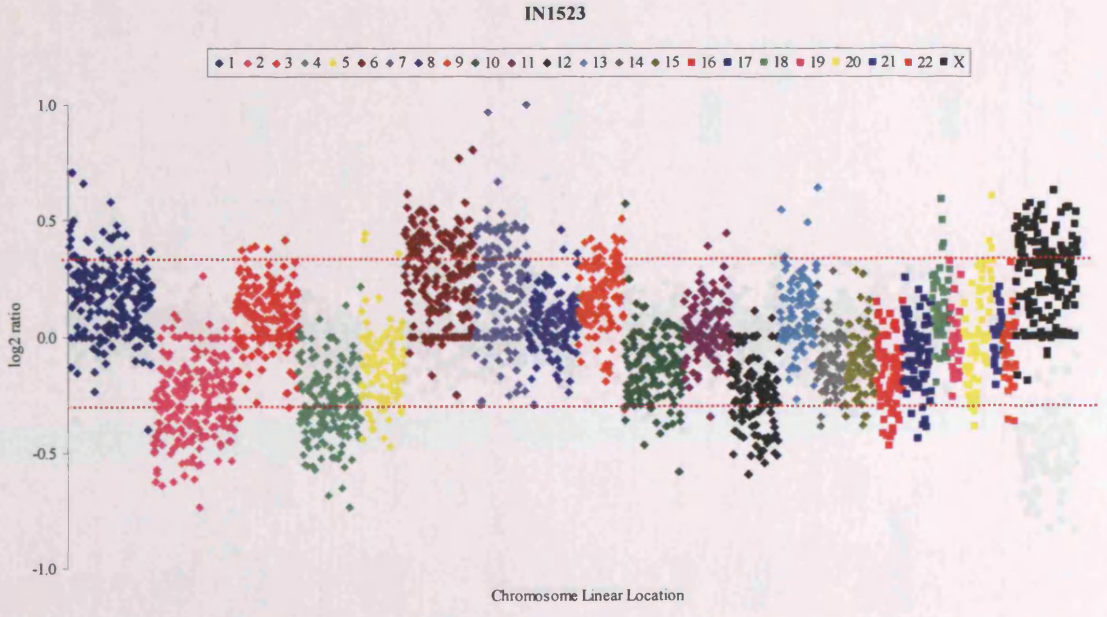
The Figure displays clone alterations across the entire genome of tumour IN1419 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.37 aCGH profile of tumour IN1495 short-term culture (GBMIV).



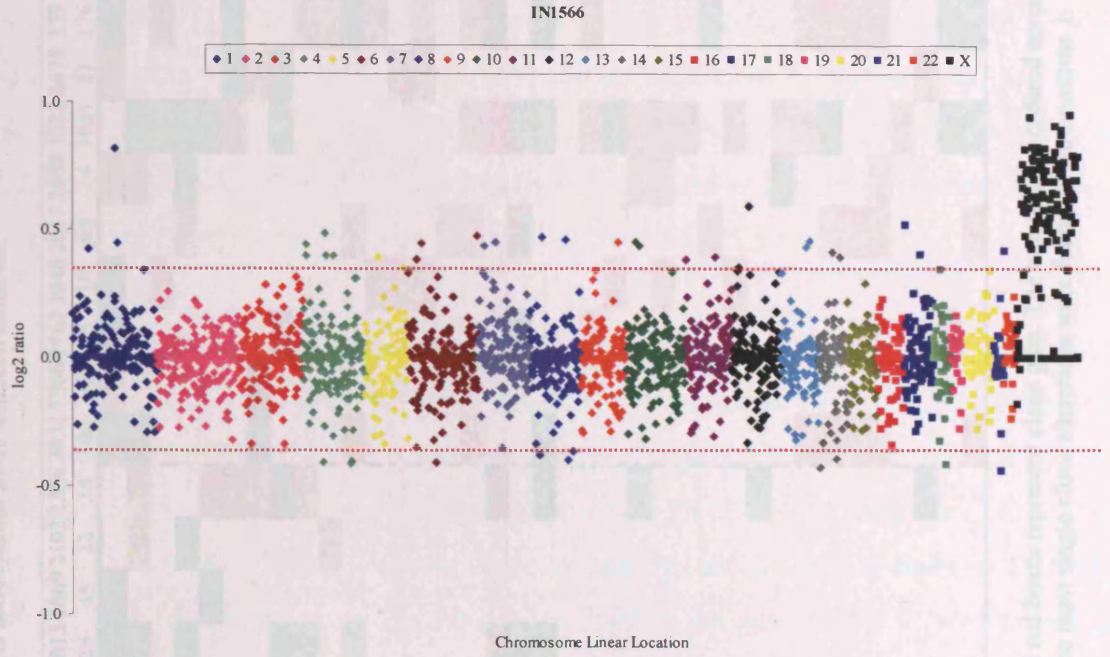
The Figure displays clone alterations across the entire genome of tumour IN1495 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.38 aCGH profile of tumour IN1523 short-term culture (GBMIV).



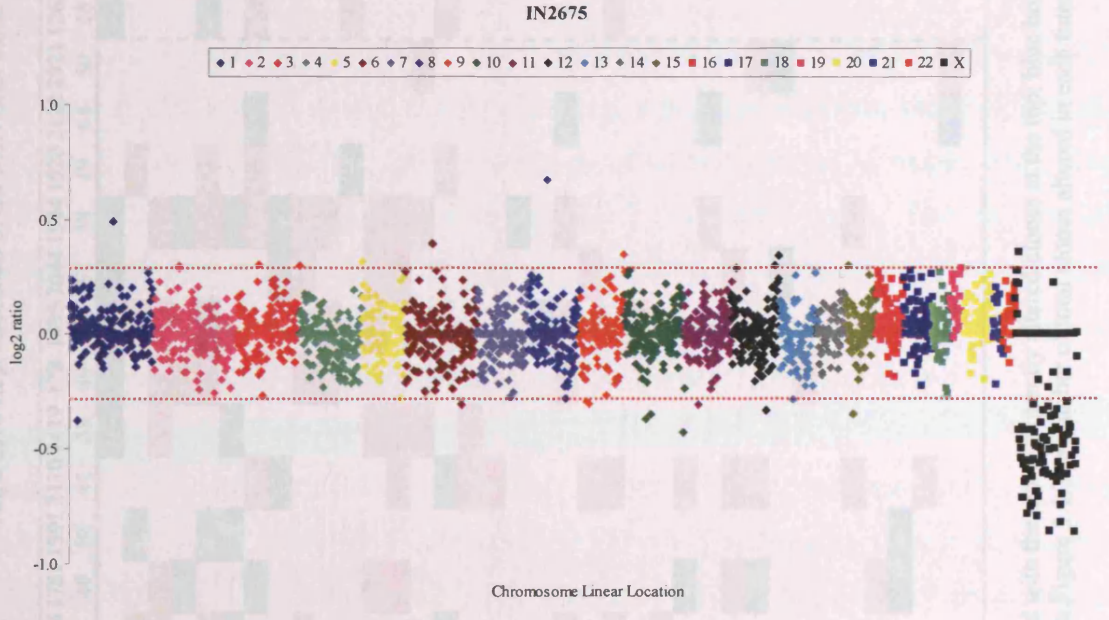
The Figure displays clone alterations across the entire genome of tumour IN1523 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.39 aCGH profile of tumour IN1566 short-term culture (GBMIV).



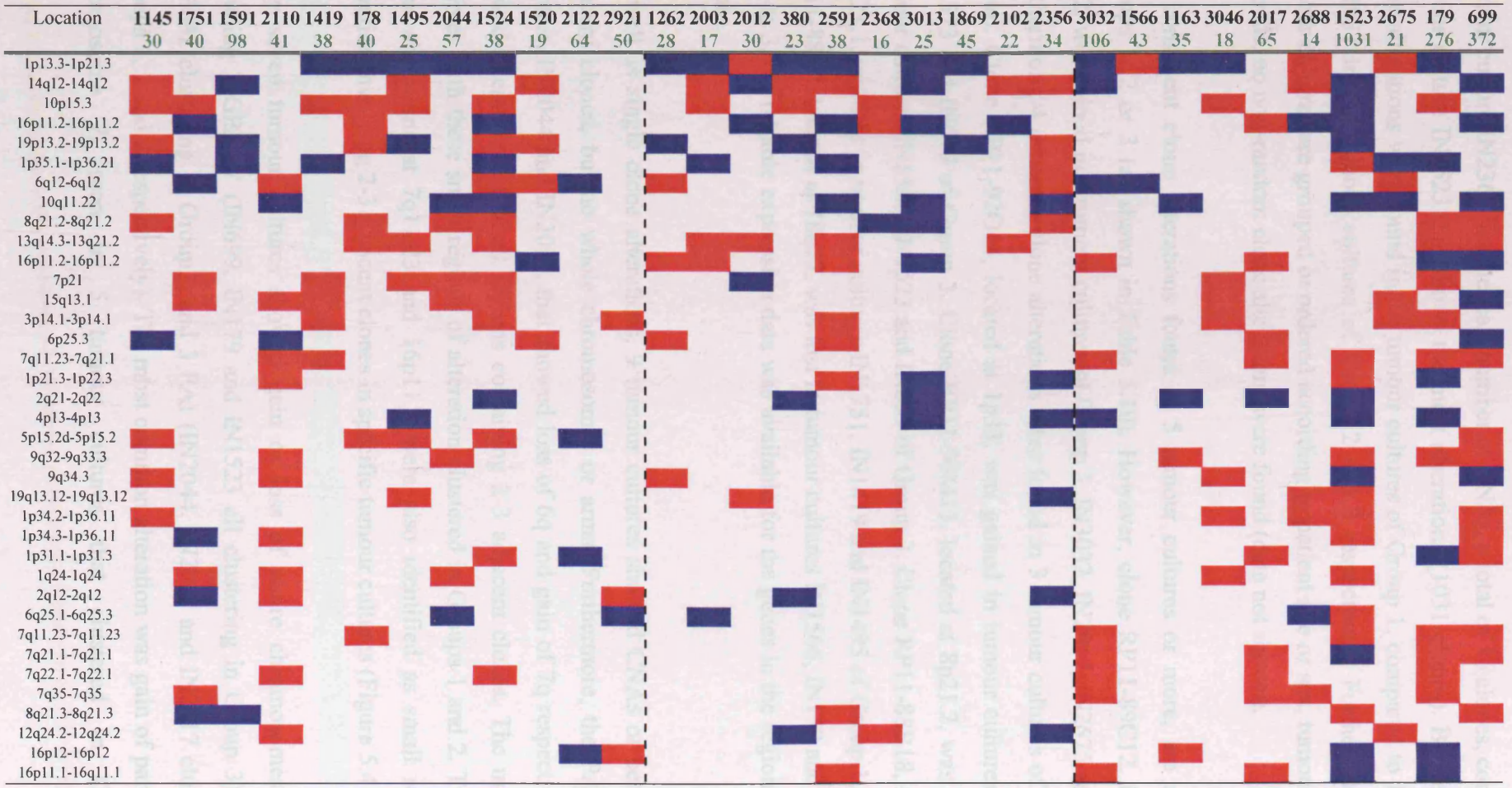
The Figure displays clone alterations across the entire genome of tumour IN1566 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Figure 5.40 aCGH profile of tumour IN2675 short-term culture (GBMIV).



The Figure displays clone alterations across the entire genome of tumour IN2675 short-term cell culture. Each chromosome is represented by a different colour and the clones that are considered to be gained or lost are those outside the red threshold lines.

Table 5.10 Representation of the individual clone CNAs found in the paediatric astrocytoma short-term cell cultures.



The Table is ordered with the most frequently altered clones at the top; blue boxes represent clone loss and red boxes represent clone gain. The tumours are ordered according to the dendrogram in Figure 5.1.The number of total clones altered in each tumour is indicated in green. The most single clone alterations were found on chromosome 1.

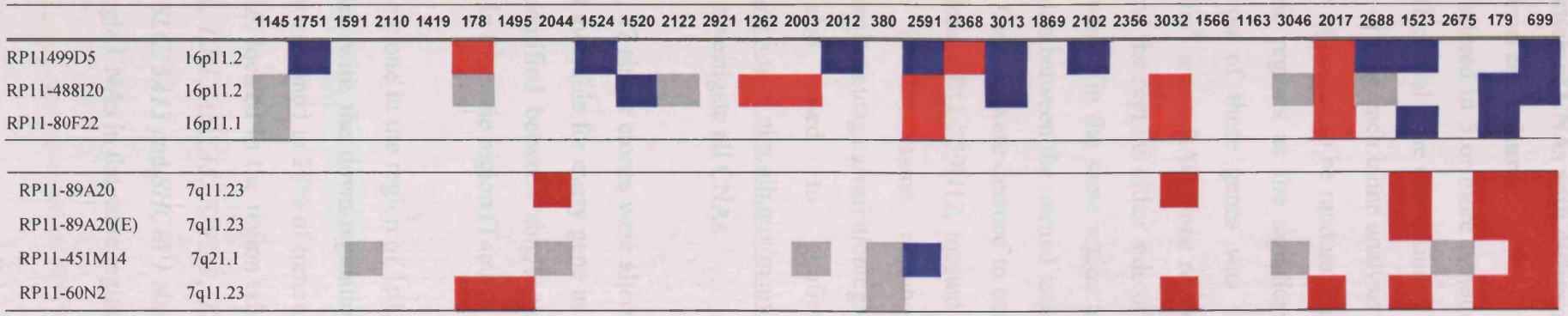
Tumour culture IN2368 has the least number of CNAs, a total of 16 clones, compared to tumour culture IN1523 that shows the most alterations (1031 clones). Between 19-98 clone alterations were found in the tumour cultures of Group 1, compared to 16-45 and 14-1031 in the tumour cultures of Group 2 and 3 respectively. Furthermore, if the tumour cultures are grouped or ordered according to patient age or sex, tumour location or grade, no non-random clone alterations were found (data not shown).

No consistent clone alterations found in 5 tumour cultures or more, are unique to Groups 1, 2 or 3 (as shown in Table 5.10). However, clone RP11-89C12, located at 2p12, was gained in 4 tumour cultures of Group 3, IN3032, IN2017, IN2675 and IN179. Furthermore, 4 different clone alterations were found in 3 tumour cultures of the same Group. Clone RP11-92O14, located at 1p33, was gained in tumour cultures IN1163, IN1523 and IN699 of Group 3. Clone RP11-90M13, located at 8p21.2, was gained in tumour cultures IN1566, IN1523 and IN699 of Group 3. Clone RP11-88B18, located at 10q21.1, was lost in tumour cultures IN1751, IN1419 and IN1495 of Group 1 and clone RP11-88B2, located at 18q22, was lost in tumour cultures IN1566, IN179 and IN699 of Group 3. No reliable expression data was available for the genes in the regions of these clones.

As well as single clone alterations, 9 tumour cultures showed CNAs of between 2-4 adjacent clones, but no whole chromosomes or arms. Furthermore, the PAI tumour cultures, IN2044 and IN2017, that showed loss of 6q and gain of 7q respectively, also showed alterations of small regions containing 2-3 adjacent clones. The majority of samples with these small regions of alteration clustered in Groups 1 and 2. Two single clone alterations at 7q11.23 and 16p11.2 were also identified as small regions of alteration involving 2-3 adjacent clones in specific tumour cultures (Figure 5.41).

Only seven tumour cultures showed gain or loss of entire chromosomes or arms including 3 GBMIV (IN699, IN179 and IN1523 all clustering in Group 3), 1 AAII (IN3032 clustering in Group3) and 3 PAI (IN2044, IN2012 and IN2017 clustering in Groups 1, 2 and 3 respectively). The most common alteration was gain of part or all of chromosome 7, seen in 5 tumour cultures that clustered in Group 3.

Figure 5.41 Clone alterations at 16p11.2 and 7q11.23 in the paediatric astrocytoma short-term cell cultures.



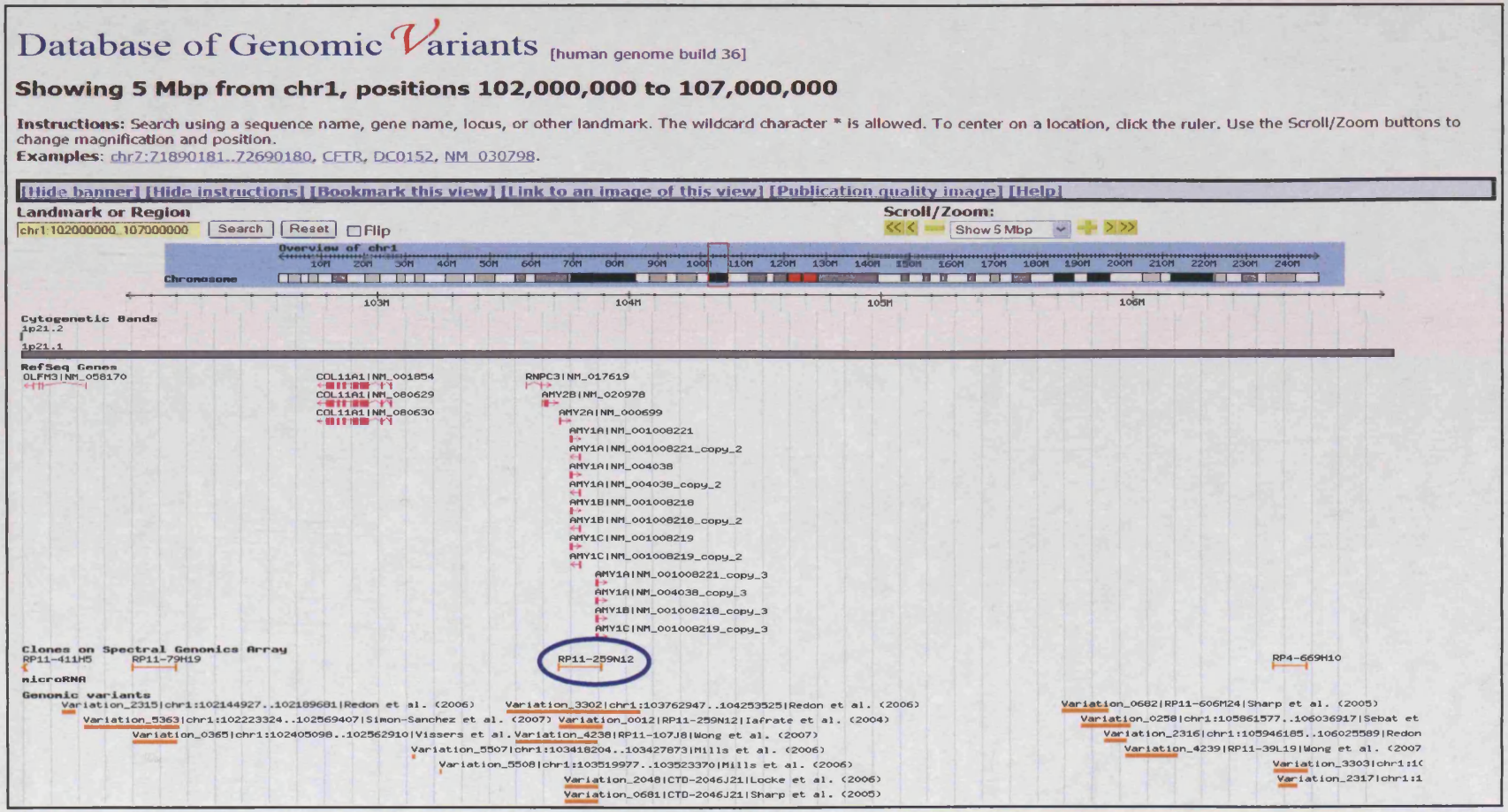
Correlation of CNAs with differential gene expression in paediatric astrocytoma short-term cell cultures

Clones altered in 5 or more tumour cultures were investigated further for correlations with differential gene expression. The cut-off was drawn here because of the extensive amount of time each clone analysis required and because the clones altered in 4 or less tumours appeared to be random (except those previously mentioned). Genes located in the same regions as the significantly altered BAC clones were identified and the expression of these genes was investigated using the data from the Affymetrix GeneChip® array. BAC clone regions were defined as the direct binding region of the clone and the regions either side of the binding region, up to each adjacent clone. Any genes located in the same region as altered clones that showed a 2-fold difference in expression between the normal astrocyte controls and all or some of the short-term cell culture Groups, were deemed to correlate with clone alterations. Figure 5.42 illustrates BAC clone RP11-259N12, located at 1p13.3-1p21.3 (103-104K), as well as the genes located in the same region using the Database of Genomic Variants (http://projects.tcag.ca/variation/cgi-bin/gbrowse/hg18). The NCBI nucleotide database was also used to confirm the genes located at 1p13.3-1p21.3 (http://www.ncbi.nlm.nih.gov/mapview/mapsearch.cgi?taxid=9606). This approach was used to investigate all CNAs.

In total, 37 single clones were altered in at least 5 tumour cultures (16%). Reliable data was not available for every gene in an altered region and consequently few correlations were identified between single clone alterations and differentially expressed genes located in the same region (Table 5.11).

A single clone in the region of 1p13.3-1p21.3 was lost in 47% of tumour cultures and correlated with the down-regulation of *COLLA1* in all cultures. A clone located at 14q12 was gained in 28% of tumour cultures and lost in 19% of cultures and the gene, *FOXG1B,* located in the region adjacent to this clone, was down-regulated in 91% of cultures. *IDI1*, *ANXA8*, *EPHA2*, *TRJ1*, *ARHGEF4*, *COL8A2*, *TRIP6*, *G10*, *ARPC1A*, *SGCE*, *SLC25A13* and *SHCBP1* also showed differential gene expression and correlated with single CNAs in the same regions.

Figure 5.42 Location of BAC clone RP11-259N12 and the genes located in the clone binding site and adjacent regions.



This was taken from the Database of Genomic variants, http://projects.tcag.ca/variation/cgi-bin/gbrowse/hg18.

Table 5.11 Single clone CNAs that correlate with differential gene expression in paediatric astrocytoma short-term cell cultures.

BAC clone	Location	Gained	Lost	% Gained	% Lost	% Total	Region size (Mbp)	No. of genes in region	Differentially expressed genes
RP11-259N12	1p13.3-1p21.3	4	15	12.50	-46.88	59.38	~4	17	↓ *COL11A1* 100%
RP11-125A5	14q12-14q12	9	6	28.13	-18.75	46.88	~1.6	6	↓ *FOXG1B* 91%
RP11-38M7	10p15.3	11	1	34.38	-3.13	37.50	~0.2	11	↑ *IDI1* Grps 2&3
RP11-499D5	16p11.2-16p11.2	3	9	9.38	-28.13	37.50	~4.3	70	
RP11-79F15	19p13.2-19p13.2	5	6	15.63	-18.75	34.38	~2.8	84	
RP1-163M9	1p35.1-1p36.21	2	8	6.25	-25.00	31.25	~0.9	32	↓ *EPHA2* 100%
RP11-80L16	6q12-6q12	6	4	18.75	-12.50	31.25	~1.5	0	
AL390716.27	10q11.22	0	10	0.00	-31.25	31.25	~4.1	85	↓ *ANXA8* 100%
RP11-96G1	8q21.2-8q21.2	7	2	21.88	-6.25	28.13	~1.3	19	
RP11-100C24	13q14.3-13q21.2	9	0	28.13	0.00	28.13	~3	6	
RP11-488I20	16p11.2-16p11.2	5	4	15.63	-12.50	28.13	~1.9	36	
CITB-13P7	7p21	7	1	21.88	-3.13	25.00	~1.3	19	
AC102941.9	15q13.1	7	1	21.88	-3.13	25.00	~3.3	43	↑ *TJP1* Grps 2&3
RP11-89A12	3p14.1-3p14.1	7	0	21.88	0.00	21.88	~1.7	11	
AL035696.14	6p25.3	4	3	12.50	-9.38	21.88	~0.2	9	
RP11-60N2	7q11.23-7q21.1	7	0	21.88	0.00	21.88	~1	3	
RP11-335D10	1p21.3-1p22.3	3	3	9.38	-9.38	18.75	~0.5	4	
RP11-89B17	2q21-2q22	0	6	0.00	-18.75	18.75	~2.5	63	↓ *ARHGEF4* 100%
RP11-81N11	4p13-4p13	0	6	0.00	-18.75	18.75	~3.3	7	
RP11-145B1	5p15.2	5	1	15.63	-3.13	18.75	~0.8	9	
RP11-9H12	9q32-9q33.3	6	0	18.75	0.00	18.75	~2.1	33	
RP1-112N13	9q34.3	4	2	12.50	-6.25	18.75	~0.2	5	
RP11-118P21	19q13.12-19q13.12	5	1	15.63	-3.13	18.75	~4.6	154	
RP5-893G23	1p34.2-1p36.11	5	0	15.63	0.00	15.63	~2.5	20	
RP1-34M23	1p34.3-1p36.11	4	1	12.50	-3.13	15.63	~2.7	38	↑ *COL8A2* Grp 2
RP11-492C3	1p31.1-1p31.3	3	2	9.38	-6.25	15.63	~1	16	
RP11-81H19	1q24-1q24	5	0	15.63	0.00	15.63	~1.1	17	
RP11-90O9	2q12-2q12	2	3	6.25	-9.38	15.63	~1.4	11	
RP11-535A9	6q25.1-6q25.3	1	4	3.13	-12.50	15.63	~1.7	11	
RP11-89A20	7q11.23-7q11.23	5	0	15.63	0.00	15.63	~0.4	3	
RP11-88D24	7q21.1-7q21.1	4	1	12.50	-3.13	15.63	~2.1	143	↑ *TRIP6* Grps 2&3, ↑ *G10* Grps 2&3, ↑ *ARPC1A* Grps 3
RP11-10D8	7q22.1-7q22.1	5	0	15.63	0.00	15.63	~7.7	143	↑ *SGCE* 100%, ↑ *SLC25A13* 100%
RP11-79M8	7q35-7q35	5	0	15.63	0.00	15.63	~4.2	25	
RP11-90O15	8q21.3-8q21.3	3	2	9.38	-6.25	15.63	~1	10	
RP11-3L23	12q24.2-12q24.2	3	2	9.38	-6.25	15.63	~3.1	44	
RP11-79I15	16p12-16p12	2	3	6.25	-9.38	15.63	~1.5	15	
RP11-80F22	16p11.1-16p11.1	3	2	9.38	-6.25	15.63	~4.3	17	↑ *SHCBP1* Grp 3

Boxed clones are located in regions of LCV in the normal population. In total 21/37 (57%) clones are located in regions of LCV.

The size of the regions investigated in this approach varied between 0.2-7.7 Mbps. To a certain extent, this explains the variation in the number of genes found in each region (0-154). Furthermore, 21 of the 37 clones investigated were located in regions of LCV that occur in the normal population.

Several tumour cultures showed CNAs of 2 or more adjacent clones in regions that correlated with differential gene expression, including whole chromosome or chromosome arm alterations (Table 5.12). In these cases, those genes that were located in the regions of alteration and showed a 2-fold difference in expression between the astrocyte controls and the tumour short-term cell cultures in which the alteration was seen, were deemed to correlate with clone alterations. Genes identified using this approach were then compared to the 4682 probe sets that were differentially expressed between the astrocyte controls, short-term cell culture Group 1, Group 2 and Group 3. Tumour culture IN699 showed the most CNAs that correlated with differential gene expression in the same regions. The majority of tumour cultures with alterations between 2 and 4 adjacent clones that correlated with differential gene expression were clustered in Group 1 or Group 2. Tumour cultures IN3032, IN2017 and IN179 are an exception to this, but they also show whole chromosome or arm alterations.

Table 5.12 The correlation of 2 or more adjacent clones in specific paediatric astrocytoma short-term cell cultures with differential gene expression where possible.

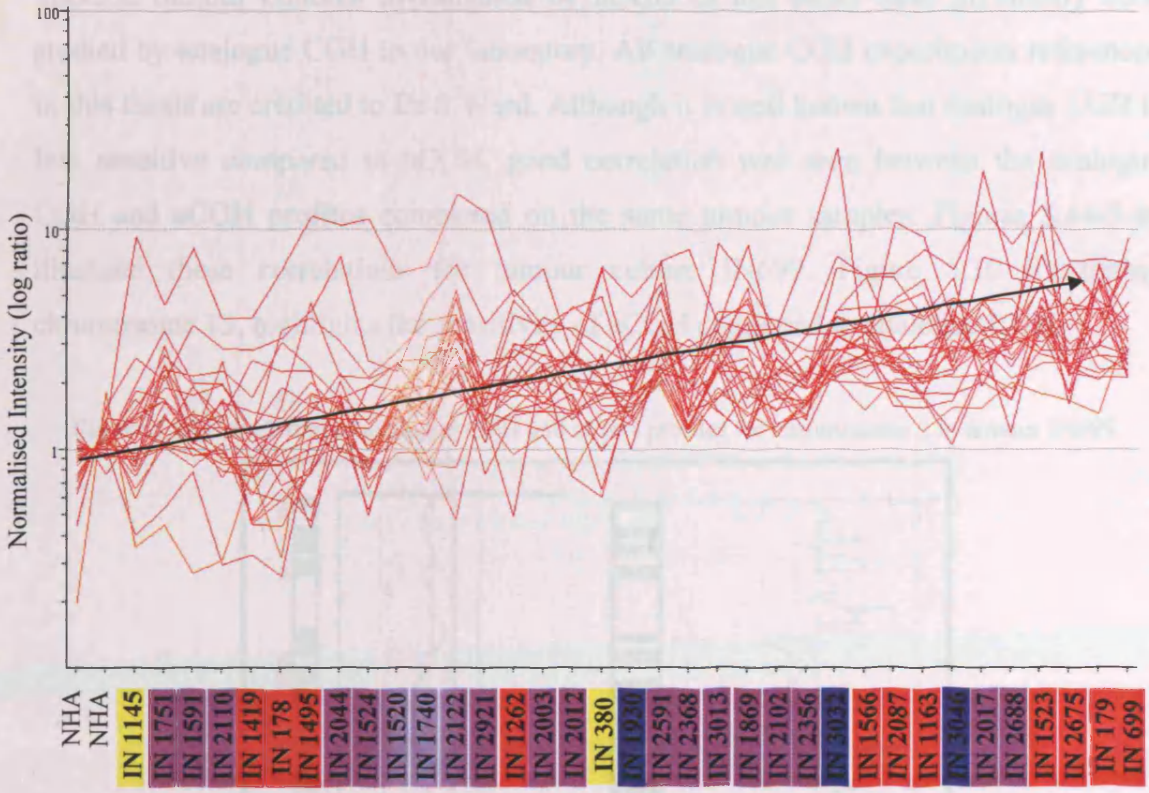
Tumour	Region	No. of clones	Loss or Gain	Region size (Mbp)	No. of genes in region	No. of differentially expressed genes
IN1145	2p13.2	2	Gain	~4.9	27	↑ *P450RAI-2*
IN2110	4q33-4q34	2	Gain	~6.5	20	↑ *FLJ20035*, ↑ *PALLD*, ↑ *NEK1*
IN178	10p15.3	2	Gain	~0.4	13	
	16p13.3	2	Gain	~3.2	165	↑ *RGS11*, ↑ *PDIA2*, ↑ *GNG13*, ↑ *ABCA3*
	22q13.1	2	Gain	~3.2	68	↑ *TCF20*, ↑ *SREBF2*, ↑ *A4GALT*
IN2044	3q24-3q25	2	Gain	~3.3	43	
	9q33	2	Gain	~3.5	42	
	6q	119	Loss	~108	653	↓ 45 (15=33%)
IN2122	4q25-4q26	2	Loss	~2.1	58	↓ NOLA1, ↓ CASP6, ↓ ENPEP, ↓ RRH, ↓ *EGF*
IN1262	3p26.3	2	Gain	~2.4	7	
	10q26.3	2	Gain	~0.2	20	
	6q12	3	Gain	~5.7	19	
IN2003	12p13.3	2	Loss	~4.2	21	↓ *PLEKHA5*
IN2012	7p14-7p21	47	Loss	~37	240	↓ 32 (22=68%)
IN2591	1p21.3	2	Gain	~1.8		
	16p11.2	2	Gain	~4.4	58	↑ *TP53TG3*
IN3013	2q14-2q22	3	Loss	~3.9	67	↓ *ARHGEF4*
	16p11.2	2	Gain	~4.4	58	↑ *TP53TG3*
IN1869	6p22.3	2	Gain	~1.2	13	
IN3032	6q26	4	Loss	~3.5	37	
	7q	71	Gain	~97	980	↑ 68 (44=64%)
	16p11.2	2	Gain	~4.4	58	↓ *TP53TG3*
	17p	30	Loss	~21	442	↓ 15 (7=46%)
IN2017	7q11.23-7qter	71	Gain	~80	980	↑ 65 (41=63%)
	16p11.2	3	Gain	~5.4	58	↓ *TP53TG3*
IN1523	2	213	Loss	~243	1888	↓ 170 (69=40%)
	4	203	Loss	~192	1154	↓ 105 (46=43%)
	6	184	Gain	~171	1505	↑ 291 (148=51%)
	7	136	Gain	~160	1452	↑ 183 (84=46%)
	9	116	Gain	~135	1148	↑ 147 (75=51%)
	12	127	Loss	~134	1370	↓ 161 (69=43%)
	20q13.2-20qter	10	Gain	~15	79	↑ 10 (4=40%)
	1p35.1-1p13.1	87	Gain	~87	730	↑ 176 (68=38%)
IN179	7	136	Gain	~160	1452	↑ 159 (84=52%)
	9	116	Gain	~135	1148	↑ 159 (78=49%)
	16p11.2	2	Gain	~4.4	58	↑ *TP53TG3*
	17q	22	Gain	~56	348	↑ 30 (18=60%)
	21	26	Gain	~47	352	↑ 32 (22=68%)
IN699	1q32-1qter	36	Loss	~52	432	↓ 31 (9=29%)
	2p23.2-2q11.2	56	Gain	~74	640	↑ 128 (45=35%)
	2q21-2q31.2	21	Gain	~41	152	↑ 53 (19=36%)
	3p14.3-3q12.2	31	Loss	~45	181	↓ 29 (16=55%)
	4	203	Loss	~192	1154	↓ 123 (55=45%)
	5pter-5p15.2	18	Gain	~36	91	↑ 19 (6=32%)
	5q33.3-5qter	14	Gain	~20	227	↑ 44 (11=25%)
	6pter-6p24	12	Loss	~7.5	70	↓ 6 (4=67%)
	7p15-7q21.1	51	Gain	~34.3	454	↑ 77 (34=44%)
	7q11.22-7q21.1	18	Gain	~14.9	124	↑ 26 (11=42%)
	8	133	Gain	~146	984	↑ 210 (96=46%)
	9pter-9q21.1	29	Loss	~26.5	145	↓ 14 (2=14%)
	9q33-9qter	21	Gain	~12.2	351	↑ 76 (24=32%)
	11q23-11qter	22	Loss	~20	177	↓ 10 (4=40%)
	13q11-13q12.3	13	Loss	~10.7	128	↓ 7 (1=14%)
	13q14-13q21.1	14	Gain	~19	116	↑ 16 (4=25%)
	13q22-13q31.1	8	Loss	~9.2	35	↓ 3 (0)
	13q31.2-13q31.3	7	Gain	~9.4	15	
	13q32.1-13qter	26	Loss	~20	214	↓ 30 (11=37%)
	15q12-15q22	40	Loss	~38.5	463	↓ 73 (31=43%)
	17p11.2-17p13.2	15	Gain	~18	236	↑ 48 (13=27%)
	18	46	Loss	~78	432	↓ 54 (23=43%)
	20q13.2-qter	9	Gain	~15	79	↑ 24 (7=29%)

The tumours are ordered according to the hierarchical clustering approach in Figure 5.1 and the dotted lines indicate tumour Groups. The percentage of genes differentially expressed in each region and also found to be differentially expressed between the astrocyte controls and the 3 tumour Groups are shown in brackets.

Gain of part or all of chromosome 7 and correlation with gene over-expression in paediatric astrocytoma short-term cell cultures

Gain of part or all of chromosome 7 was the most common chromosomal alteration found in 5 paediatric astrocytoma short-term cell cultures, comprising 3 GBMIV (IN699, IN1523 and IN179), 1 AAII (IN3032) and 1 PAI (IN2017). Gain of part or all of this chromosome correlated with a greater than 2-fold up-regulation of 103, 183, 159, 68 and 65 genes in tumour cultures IN699, IN1523, IN179, IN3032 and IN2017 respectively (Table 5.12). In total, 16 genes located on chromosome 7 showed a common 2-fold up-regulation in the 5 tumours (Figure 5.43). Furthermore, the expression profile of these 16 genes in all tumours shows a continuous increase in up-regulation from those tumours in Group 1 to those of Group 3.

Figure 5.43 The expression profile of the 16 genes that show a 2-fold increase in expression in all paediatric astrocytoma short-term cell cultures with gain of chromosome 7.



The tumours are ordered according to the hierarchical clustering diagram in Figure 5.1.

Table 5.13 details the 16 genes, including those highlighted red, which have functions previously investigated in astrocytoma or that have been linked to tumour or cancer development.

Table 5.13 Details of the 16 genes that show a greater than 2-fold increase in expression in all samples with gain of part or all of chromosome 7.

Location	Common Name	Description
Chr:7q11.21	FLJ10099	hypothetical protein FLJ10099
Chr:7q11.23	POMZP3	POM (POM121 homolog, rat) and ZP3 fusion
Chr:7q21.1	*SRI*	*sorcin*
Chr:7q21.2	FLJ20073	hypothetical protein FLJ20073
Chr:7q21.3	SLC25A13	solute carrier family 25, member 13 (citrin)
Chr:7q21-q22	SGCE	sarcoglycan, epsilon
Chr:7q22.1	*PBEF1*	*pre-B-cell colony-enhancing factor*
Chr:7q22.1	SYPL	synaptophysin-like protein
Chr:7q31.1-q31.3	LSM8	LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae)
Chr:7q31.2-q31.3	*CAPZA2*	*capping protein (actin filament) muscle Z-line, alpha 2*
Chr:7q31-q32	DLD	dihydrolipoamide dehydrogenase
Chr:7q31-q32	GNG11	guanine nucleotide binding protein (G protein), gamma 11
Chr:7q32	CALU	calumenin
Chr:7q32.3	CHCHD3	hypothetical protein FLJ20420
Chr:7q36	*ABCF2*	*ATP-binding cassette, sub-family F (GCN20), member 2*
Chr:7q36.3	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6

Detailed aCGH analysis

Specific tumour cultures investigated by aCGH in this study have previously been studied by analogue CGH in our laboratory. All analogue CGH experiments referenced in this thesis are credited to Dr S Ward. Although it is well known that analogue CGH is less sensitive compared to aCGH, good correlation was seen between the analogue CGH and aCGH profiles completed on the same tumour samples. Figures 5.44-5.46 illustrate these correlations for tumour culture IN699. Figure 5.36 illustrating chromosome 13, highlights the sensitivity of aCGH compared to analogue CGH.

Figure 5.44 Correlation of analogue CGH and aCGH profiles for chromosome 3 in tumour IN699.

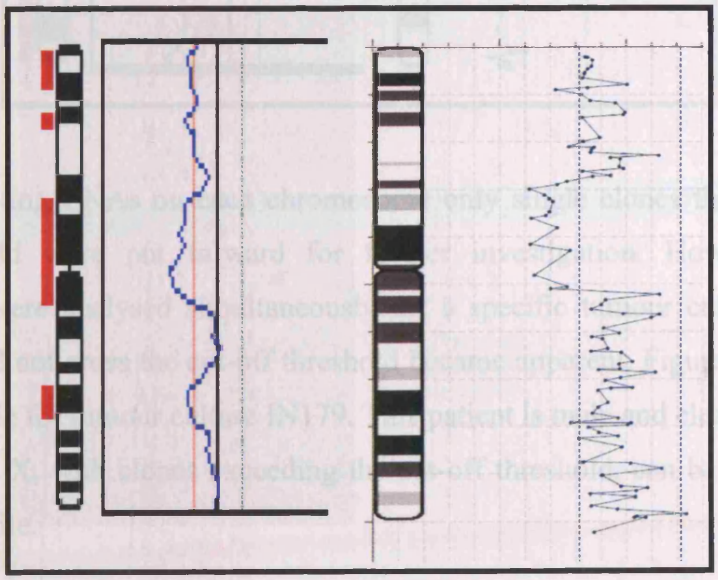


Figure 5.45 Correlation of analogue CGH and aCGH profiles for chromosome 9 in tumour IN699.

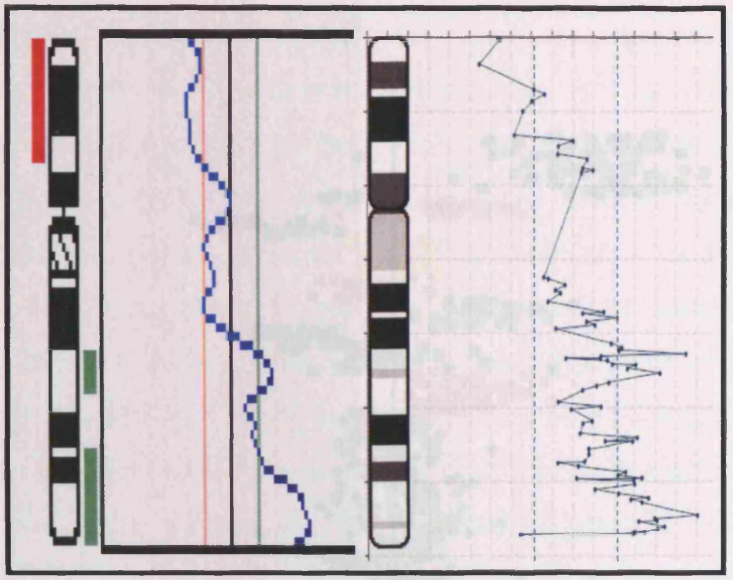
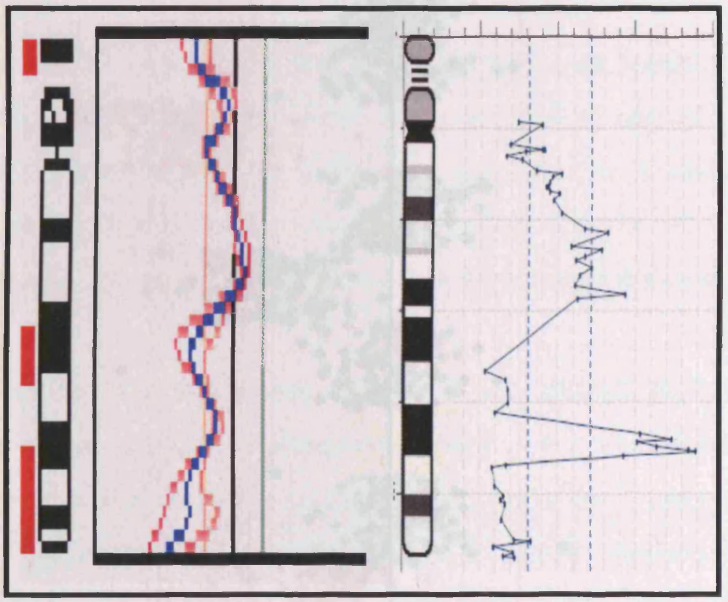
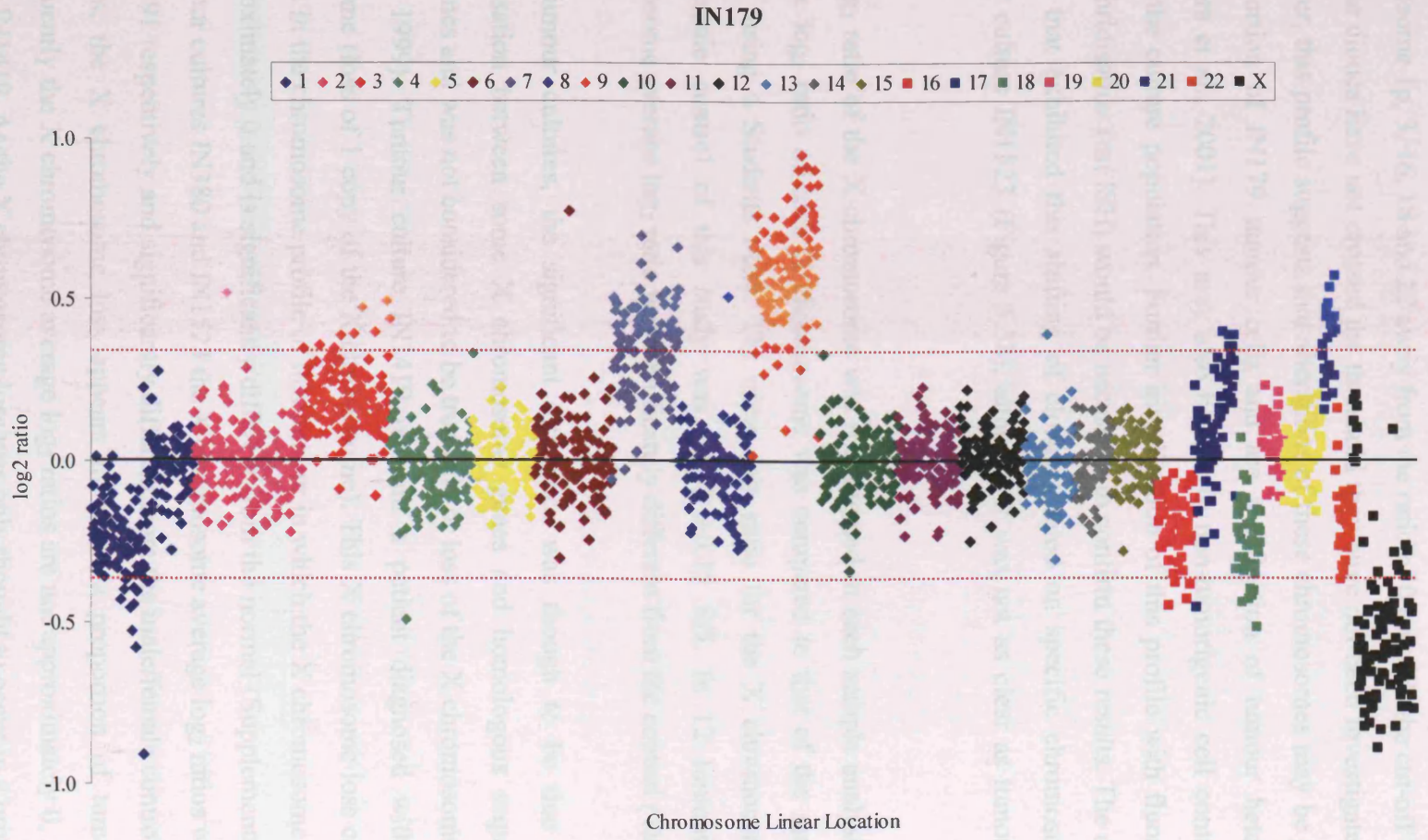


Figure 5.46 Correlation of analogue CGH and aCGH profiles for chromosome 13 in tumour IN699.



When investigating CNAs on each chromosome only single clones that exceeded the cut-off threshold were put forward for further investigation. However when all chromosomes were analysed simultaneously for a specific tumour culture, additional changes that did not cross the cut-off threshold became apparent. Figure 5.47 illustrates the aCGH profile for tumour culture IN179. This patient is male and clear loss of 1 copy of chromosome X, with clones exceeding the cut-off threshold, can be seen on the far right of the profile.

Figure 5.47 Tumour IN179 aCGH profile for all chromosomes.



The single clones on each chromosome are represented by a spot, the spots for each chromosome are coloured and are ascending from left to right with the X chromosome on the far right. The cut-off threshold for clone gains and losses is represented by the dotted red line.

The alterations documented in Table 5.12 can also be seen in the profile including, gain of 1 copy of chromosome 7, gain of possibly 2 copies of chromosome 9, gain of 1 copy of 17q and gain of 1 copy of 21. Moreover, there is a clear shift in the clones located on chromosome 1p, 3, 16, 18 and 22 away from the ratio of 0 towards the cut-off threshold. As these clones have not crossed the threshold they have not been investigated further. However, this profile suggests that alterations of these chromosomes may be present in a proportion of IN179 tumour cells and are a reflection of tumour heterogeneity (Snijders et al., 2001). This may also be due to non-tumorigenic cell contamination within the culture population. Further investigation of this profile with fluorescent *in situ* hybridisation (mFISH) would be necessary to confirm these results. The only other profile that exhibited this shifting of clones located on specific chromosomes was tumour culture IN1523 (Figure 5.38), although it was not as clear as tumour culture IN179.

The $\log_2$ ratio of the X chromosome was investigated in each sample analysis and the average $\log_2$ ratio of each X chromosome was compared to that of the male/female control using a Students t-test. The mean $\log_2$ ratio for the X chromosome in the male/female control of this study was ±1.41 ±0.15 SD. In 12 tumours, the X chromosome average $\log_2$ ratio was significantly different from the control (Table 5.14).

In 9 tumour cultures, the significant difference was though to be due to cross-hybridisation between some X chromosome genes and homologous sequences on autosomes and was not considered to be true gain or loss of the X chromosome (Pollack et al., 1999). Tumour culture IN1419 is from a patient diagnosed with Turners Syndrome (loss of 1 copy of the X chromosome). This X chromosome loss can clearly be seen in the chromosome profile of this tumour in which the X chromosome $\log_2$ ratio is approximately 0 and is significantly different from the normal (Supplementary Data). In tumour cultures IN380 and IN1523 the X chromosome average $\log_2$ ratios were 0.327 and 0.291 respectively and significantly different from the male/female control. In these tumours, the X chromosome loss appears to be in a proportion of tumour cells, consequently the X chromosome average $\log_2$ ratios are not approximately 0, as seen in tumour IN1419. As the X chromosome loss was only thought to occur in a proportion of tumour cells, differential gene expression on the X chromosome was not directly

investigated in these tumours. This may also be due to non-tumorigenic cell contamination in the cell culture sample.

Table 5.14 The X chromosome $\log_2$ ratio averages and SD for 12 tumours that showed a significant difference from the male/female normal control.

Tumour	Significance	X chromosome average $\log_2$ ratio and SD
IN1419	**$p < 0.001$*****	**0.053 ±0.212**
IN1524	$p < 0.05$	-0.554 ±0.206
IN1520	$p < 0.01$**	-0.559 ±0.214
IN1262	$p < 0.001$***	-0.560 ±0.209
IN380	**$p < 0.001$*****	**0.327 ±0.203**
IN2591	$p < 0.05$*	0.554 ±0.2122
IN3013	$p < 0.05$*	-0.554 ±0.220
IN1869	$p < 0.01$**	0.557 ±0.241
IN1566	$p < 0.001$**	0.591 ±0.219
IN1163	$p < 0.01$**	-0.560 ±0.242
IN2017	$p < 0.01$**	-0.555 ±0.230
IN1523	**$p < 0.001$*****	**0.291 ±0.163**

Results shown in bold suggest true X chromosome alterations.

DISCUSSION

Array technology has been used to define genomic aberrations and generate gene expression profiles of paediatric astrocytoma short-term cell cultures with the main aims of correlating differential gene expression and genomic aberrations in paediatric astrocytoma and characterising disrupted cellular pathways that could be involved in tumour development and growth. Genomic gain and loss has been shown as a mechanism that can disrupt gene expression and play a significant role in the development of tumours.

The review of tumour diagnoses in this Chapter, according to 2007 WHO grading scheme, revealed some unexpected results. Two tumours, IN1930 and IN3032, previously diagnosed as DAII were re-graded as AAIII and all remaining DAII were re-

graded as PAI. In the experience of the pathologists involved in this review, paediatric astrocytoma are rarely classified as DAII. In this study, it was not sensible to analyse the tumour cultures by pathological grade due to the clustering patterns seen, highlighting the difficulties of analysing paediatric astrocytoma by pathological grade. The 2007 WHO grading scheme, does show a higher correlation with the 3 tumour Groups seen here, with Groups 1 and 2 consisting of predominately PAI cultures and Group 3 consisting of mostly high-grade tumour cultures. Furthermore, this suggests that two sub-groups of PAI do exist as seen by Wong et al. (2005), although different genes in this study characterise the sub-groups, as seen by the investigation of Groups 1 and 2. However, the tumour samples of this study are short-term cultures compared to biopsies used by Wong et al. (2005) and this may explain why different genes characterise the two PAI sub-groups in each study. Moreover, the PA biopsies investigated in Chapter 3 do not show clear sub-groups, but the tumours that cluster furthest apart do show characteristics of each sub-group identified by Wong et al. (2005). Additional biopsy samples maybe need to be added to this cohort to distinguish any subgroups.

Differential gene expression and cell signalling pathways in paediatric astrocytoma short-term cell cultures

In total, 4682 probe sets showed a 2-fold and significant change in expression between the 3 tumour culture Groups and normal controls. k-means clustering allowed the grouping of genes according to expression profile similarity and identified molecular signatures that could define each tumour culture Group. Sets 7 and 10 of the clustering approach identified genes that were expressed at a low level in all Groups compared to the controls and may be characteristic of astrocytoma. Clusters sets 4, 5 and 6 were differentially expressed in Groups 2 and 3 compared to Group 1 and controls and could be used to distinguish these tumour culture clusters. Sets 3 and 8 were differentially expressed in Group 1 and 2 tumour cultures compared to Group 3 and the controls and could distinguish Group 1 and 2 tumour cultures. Sets 2 and 9 were differentially expressed in Group 2 tumour cultures only and set 1 was differentially expressed in Group 3 tumour cultures only. These sets could be used to characterise these specific tumour culture clusters. Overall each tumour Group has a specific molecular signature that distinguishes the clusters and the controls. This is most relevant with respect to Group 3 tumour cultures that show increased CNAs (discussed later in this Chapter).

The cluster stets of this k-means approach were too large to investigate by data mining, consequently Onto-tools was used to identify biological processes and pathways that were significantly altered in each tumour Group.

The characterisation of the biological pathways significantly disrupted in the different clusters of paediatric astrocytoma, highlighted that the tumour cultures of Group 3 show a larger number of differentially expressed genes compared to Groups 1, 2 and the controls. Overall, only the biological process antigen processing and presentation was significantly altered in all 3 Groups. Cell motility, cell growth, angiogenesis, cell adhesion and phosphate transport were altered in Groups 1 and 2 and ER to golgi vesicle transport, attachment of glycophosphatidylinositol (GPI) anchor protein, electron transport and barbed-ended actin filament capping were altered in Groups 2 and 3. Protein biosynthesis was found to be altered in Group 3, with specific biosynthesis processes, such as glutathione biosynthesis, also showing alterations in the tumours of Group 1. Furthermore, cell differentiation was only significantly altered in Group 1 tumour cultures.

Although the up-regulation of genes involved in immune response has been reported in PAI compared higher grade astrocytomas as discussed in Chapter 4 (Hunter et al., 2002; Huang et al., 2005), reduced staining of immune response genes have also been reported in high-grade astrocytoma compared to normal brain (Facoetti et al., 2005). Further investigation of immune response genes in paediatric astrocytoma is necessary to fully understand this pathway. The majority of significantly altered biological processes common to Groups 2 and 3 are characteristic of tumour development. However, several of the processes unique to Group 3 are not well characterised in paediatric astrocytoma. Glycosyl-phosphatidylinositol (GPI) anchor protein up-regulation has been linked to increased tumourigenesis and invasion in breast cancer (Wu et al., 2006). Furthermore, GPI anchor attachment protein 1 (GPAA1), located at 8q24.3, was found to be frequently up-regulated in hepatocellular carcinomas (Ho et al., 2006). Moreover, the control of barbed-ended actin filament capping regulates actin filament polymerisation and lamellipodia formation influencing cell migration (Carlier et al., 2003). Cell differentiation was also identified as a significantly altered biological process in PAI by Wong et al. (2005).

The impact of differential gene expression on cellular pathways was assessed by Onto-tools for each cluster Group. The ranking of the focal adhesion and ECM-receptor interaction pathways as 1 or 2 in each tumour culture cluster is most likely a reflection of the samples being derived short-term cell cultures. All ranked pathways showed variations between the 3 tumour culture Groups, with the Wnt signalling and the cell cycle pathways illustrating differential gene expression in all Groups previously associated with tumour development (Figures 5.3 and 5.4). The hypothesis stating that the disruption of the cell cycle and Wnt signalling through differential gene expression in each Group to different extents was true. With the number of differentially expressed genes being greater in the more malignant tumour cultures of Group 3.

Wnt signalling has a role in both embryological development and mature tissues controlling cell proliferation, fate and self-renewal. Three different pathways can be activated by Wnt signalling molecules, including the canonical Wnt signalling pathway, the Wnt/planar cell polarity pathway and the Wnt/calcium pathway. This report will focus on the canonical Wnt signalling pathway which influences the stabilisation of β-catenin transcription activity and is most relevant in cancer development.

Alterations in many genes of the Wnt signalling pathway have been identified in a wide variety of tumours and cancer (Table 5.15). Signalling in this pathway is tightly regulated and controlled by several interacting mechanisms. Inhibition can occur through sequestration of Wnt ligands from Frizzled receptors by WIF-1 or Cer-1 (Hsieh et al., 1999; Piccolo et al., 1999) or through LRP5 and LPR6 receptor inactivation by the Dickkopf (Dkk) family of proteins via endocytosis (Mao et al., 2001). Expression data was not available for *WIF-1* or *Cer-1*. However up-regulation of *LRP5* and *LRP6* as well as *Dkk* was seen in both Group 1 and 2 tumour cultures. Furthermore, Wnt ligands and frizzled receptors were down-regulated in each tumour Group.

Degradation of β-catenin through independent and dependent phosphorylation controls cellular protein levels. Degradation of β-catenin through dependent phosphorylation requires recruitment to a large complex consisting of Axin, APC, GSK3β, CKIα and PP2A, ultimately leading to ubiquitination and protein destruction by the proteasome system (Ciechanover, 1998; Hinoi et al., 2000). Independent degradation involves TP53 induction of Siah1 which forms a complex with β-catenin and APC, promoting

ubiquitination of β-catenin without GSK3β phosphorylation (Liu et al., 2001). Furthermore, pathway receptor activity is propagated through the dishevelled protein that inhibits GSK3β activity preventing β-catenin degradation.

Table 5.15 Wnt associated genes previously reported in other types of tumour and cancer with differential expression and/or mutation. Taken from Ilyas., (Ilyas, 2005).

Wnt signalling component	Change	Cancer Type
Wnts	Over-expression	NSCLC, CLL, Gastric cancer, HNSCC, Colorectal, Ovarian, BCC
	Down-regulation	Breast
sFRPs	Down-regulation	Breast, Bladder, Mesothelioma, Colorectal
	Over-expression	Prostate
WIF-1	Down-regulation	NSCLC, Prostate, Breast, Lung, Bladder
LRP5	Over-expression	Osteosarcoma
Frizzled	Over-expression	Prostate, HNSCC, Colorectal, Ovarian, Oesophageal, Gastric
Dishevelled	Over-expression	Prostate, Breast, Mesothelioma, Cervix
Frat1	Over-expression	Pancreatic, Oesopahgeal, Cervical, Breast, Gastric
CTNNB1	GOF mutation	Gastric, Colorectal, Intestinal carcinoid, Ovarian, Pulmonary adenocarcinoma, Endometrial, Hepatocellular, Hepatoblastoma, Medulloblastoma, Pancreatic, Thyroid, Prostate, Melanoma, Pilomatricoma, Wilms tumour, Pancreatoblastoma, Liposarcoma, Juvenile nasoparyngeal angiofibromas, Desmoia, Synovial sarcoma
APC	LOF mutation	Colorectal, Melanoma, Medulloblastoma, Desmoid
Axin	LOF Mutation	Hepatocellular, Medulloblastoma

NSCLC=non-small cell lung cancer; HNSCC=head and neck squamous cell carcinoma; CLL=chronic lymphatic leukaemia; BCC=basal cell carcinoma; GOF=gain of function; LOF=loss of function

Most β-catenin gene (*CTNNB1)* mutations are found in exon 3, leading to the loss of the GSK3β recognition motif. This prevents phosphorylation and degradation leading to increased protein concentration (Morin et al., 1997). Mutations of *APC* or *Axin* inhibiting β-catenin degradation have also been documented suggesting loss of β-catenin regulation is selective for mutations in proteins involved in degradation (Segditsas and Tomlinson, 2006). *GSK3β* and *PP2A* were expressed at normal levels in all tumour Groups; up-regulation of *Axin* was seen in all tumour Groups and *CKIα* in Groups 2 and 3. The down-regulation of the *dishevelled* gene (*Dv1*) was also found in tumour Groups 2 and 3 as well as *Siah-1* down-regulation in Group 3 tumour cultures. Moreover, the up-regulation of *CTNNB1* and down-regulation of *APC* was only found in the tumour cultures of Group 3 suggesting increased pathway activity. Increased β-

catenin immunoreactivity has previously been linked to increased astrocytoma grade and poor prognosis (Utsuki et al., 2002).

To activate transcription, β-catenin is translocated to the nucleus where it complexes with members of the LEF/TCF family of proteins. These proteins serve as a β-catenin DNA binding domain as β-catenin itself can not directly bind DNA (Behrens et al., 1996). Members of this transcription family were up-regulated in all tumour culture Groups.

The disruption of Wnt signalling through elevated levels of *CTNNB1* results in constant activation or inhibition of a large number of target genes that may affect cellular processes linked to tumour development (Figure 5.48). In this study, target genes *KLF4* and *CD44* were up-regulated in all tumour cultures; *PLAUR* was down-regulated in tumour culture Groups 1 and 2; *PPARD* was down-regulated in tumour culture Groups 2 and 3; and *survivin* was up-regulated in Group 3 tumour cultures. Over-expression of survivin has been correlated with poor prognosis in high-grade astrocytoma and inhibition of gene expression has been shown to induce caspase-dependent apoptosis (Blum et al., 2006; Saito et al., 2007).

Disruption of Wnt signalling has a huge impact on cellular function. Moreover, with Group 3 tumour cultures showing both up-regulation of *CTNNB1* and down-regulation of *APC,* it is possible to suggest that disruption of the Wnt signalling pathway in these tumours promotes tumour development. Although Groups 1 and 2 tumour cultures do not demonstrate differential expression in as many genes of the Wnt signalling pathway as Group 3 tumour cultures, pathway signalling is still altered influencing tumour development.

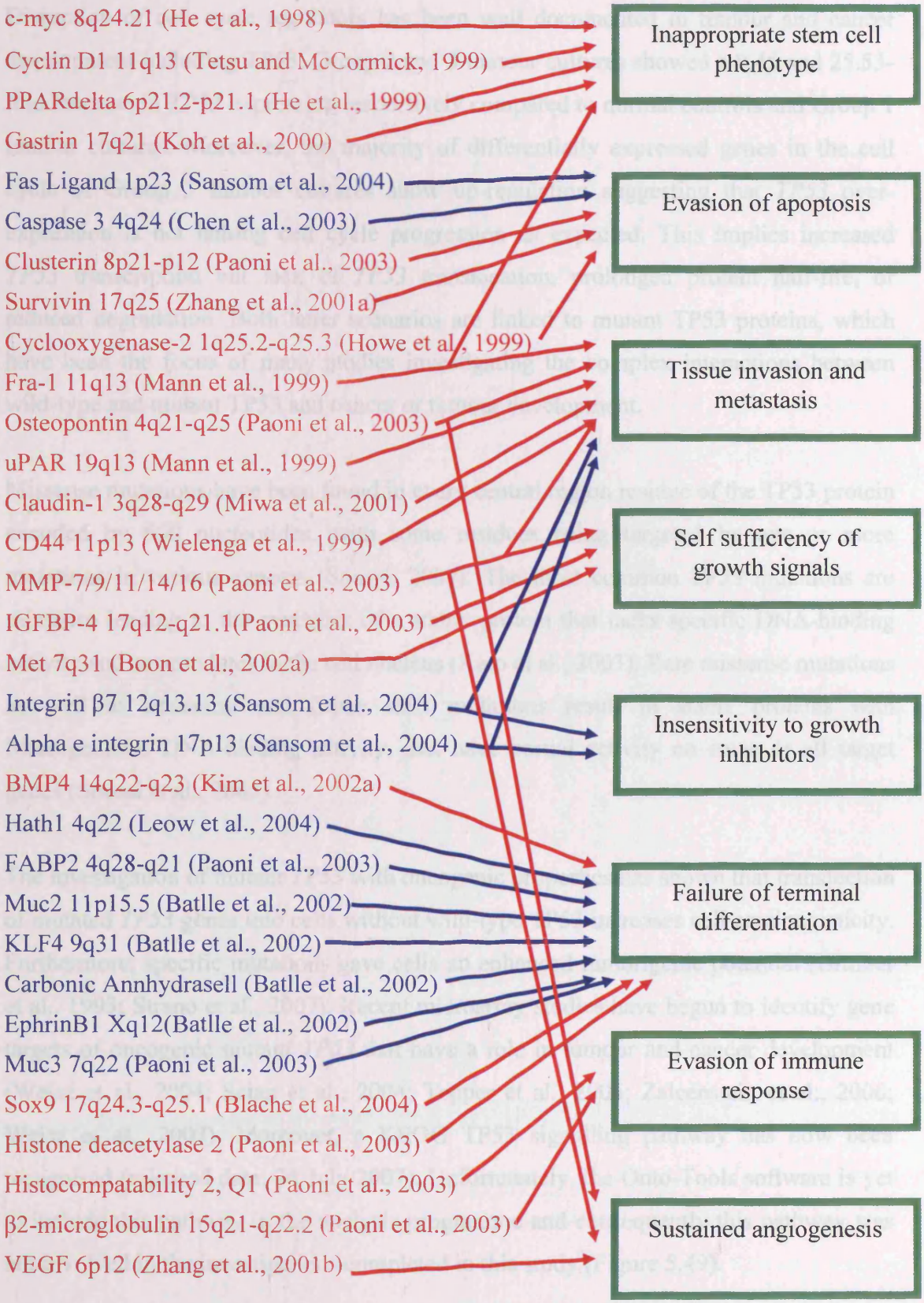


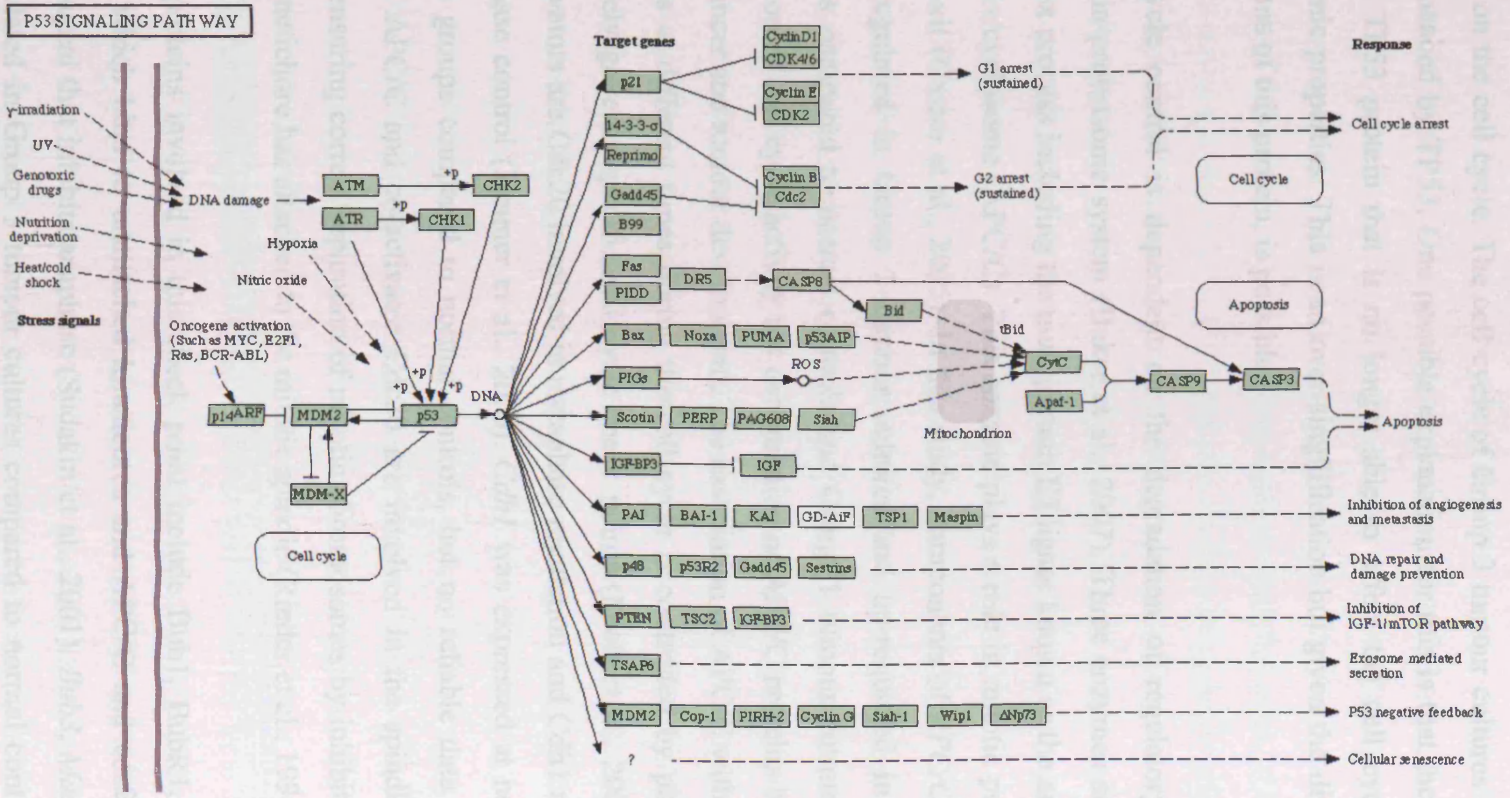
Figure 5.48 This Figure illustrates genes that are affected by elevated β-catenin levels through disrupted Wnt signalling causing continuous expression or inhibition. These genes affect a large number of cellular processes that can be associated with tumour development and progression. Up-regulated genes are shown in red and down-regulated genes are shown in blue. Adapted from Ilyas., (Ilyas, 2005).

Disruption of cell cycle regulators has been well documented in tumour and cancer development including TP53. Group 2 and 3 tumour cultures showed a 9.45 and 25.53-fold increase in *TP53* expression respectively compared to normal controls and Group 1 tumour cultures. Moreover, the majority of differentially expressed genes in the cell cycle of Group 3 tumour cultures show up-regulation suggesting that *TP53* over-expression is not halting cell cycle progression as expected. This implies increased *TP53* transcription but lack of *TP53* translocation, prolonged protein half-life, or reduced degradation. Both latter scenarios are linked to mutant TP53 proteins, which have been the focus of many studies investigating the complex interactions between wild-type and mutant TP53 and cancer or tumour development.

Missense mutations have been found in every central region residue of the TP53 protein encoded by 600 nucleotides, with some residues being targeted by one or more mutations in various cancers (Soussi, 2007). The most common *TP53* mutations are missense leading to the synthesis of a stable protein that lacks specific DNA-binding activity and accumulates in the cell nucleus (Kato et al., 2003). Rare missense mutations as well as nonsense and frame shift mutations result in stable proteins with heterogeneous DNA-binding activity, that have partial activity on some or all target genes (Soussi et al., 2005).

The investigation of mutant *TP53* with oncogenic properties has shown that transfection of mutated *TP53* genes into cells without wild-type TP53 increases cell carcinogenicity. Furthermore, specific mutations gave cells an enhanced tumorigenic potential (Dittmer et al., 1993; Strano et al., 2007). Recent microarray studies have begun to identify gene targets of oncogenic mutant *TP53* that have a role in tumour and cancer development (Weisz et al., 2004; Scian et al., 2004; Tepper et al., 2005; Zalcenstein et al., 2006; Weisz et al., 2007). Moreover, a KEGG TP53 signalling pathway has now been recognised (released date; 24 July 2007). Unfortunately, the Onto-Tools software is yet to include this pathway in the analysis programme and consequently this pathway was not involved in the investigations completed in this study (Figure 5.49).

Figure 5.49 The KEGG TP53 signalling pathway



In this study, the cell cycle genes of Group 2 tumour cultures are down-regulated compared to Group 1 tumour cultures and normal controls suggesting that in these tumour cultures the TP53 protein is functional or is a mutant protein that is able to exert effects on the cell cycle. The cell cycle of Group 3 tumour cultures does not appear to be influenced by TP53. One possible explanation for this is that these tumours have a mutant TP53 protein that is no longer able to affect the cell cycle and may have oncogenic properties. This is an over-simplification but given the diverse function and mutations of this protein, is possible.

Cell cycle control is dependent on the degradation of regulatory proteins by the ubiquitin-proteasome system (Baker et al., 2007). Three enzymes are involved in this complex process including the multiprotein E3 ligase known as the anaphase promoting complex/cyclosome (APC/C). This enzyme plays a role in mitotic progression and cell cycle exit (Castro et al., 2005). In this study, components of *APC/C* were found to be down-regulated in Group 2 tumour cultures and up-regulated in Group 3 tumour cultures compared to normal controls and Group 1 tumour cultures. This may be a reflection of cell cycle activity but deregulation of APC/C proteins has been associated with cancer and tumour development. The association of APC/C with different activator proteins at different times during the cell cycle is controlled by phosphorylation and ultimately governs specific cell cycle check points (Kraft et al., 2003). The two main co-activators are Cdc20 involved in metaphase regulation and Cdh1 responsible for late metaphase control (Kramer et al., 2000). *Cdh1* was expressed at normal levels in all tumour groups compared to normal controls, but no reliable data was available for *Cdc20*. APC/C and co-activator Cdc20 are involved in the spindle assembly check point, ensuring correct separation of mitotic chromosomes by inhibiting anaphase until each kinetichore has attached to the mitotic spindle (Rieder et al., 1994; Yu, 2002).

Other proteins involved in this check point include Bub1, BubR1, Bub3, Mad1 and Mad2, which bind to unattached kineticores and APC/C and cdc20 to generating a 'stop' signal that inhibits anaphase (Sudakin et al., 2001). *Bub3*, *Mad1* and *Mad2* were up-regulated in Group 3 tumour cultures compared to normal controls, Group 1 and Group 2 tumour cultures. This is contradictory to the up-regulation of *APC/C* and the overall increased cell cycle activity seen in this Group, suggesting that the spindle check point is failing to halt cell cycle progression.

Dysfunction of the spindle checkpoint has been proposed as a cause of chromosome instability resulting in aneuploidy (Cahill et al., 1998). Mutations in mitotic checkpoint genes themselves have been identified as a mechanism of checkpoint impairment in human tumour cells (Ohshima et al., 2000; Ru et al., 2002). Furthermore, some aneuploid cell lines do not show mutations in spindle check point genes but do show alterations in gene expression (Bharadwaj and Yu, 2004). *Mad2* haploinsufficiency in mice correlated with a 30% reduction in protein level that lead to spindle check point deficiencies and a high incidence of lung tumours (Michel et al., 2001). It is clear that small alterations in the expression of spindle checkpoint genes or mutations can disrupt the check point influencing chromosome stability and aneuploidy. Although still to be discussed in detail, the tumour cultures of Group 3 showed increased whole chromosome CNAs providing further evidence for spindle check point disruption in this tumour group.

DNA replication involves the precise duplication of the entire genome once per cell cycle. Each chromosome contains starting points of replication or origins of replication. These origins are tightly regulated, only becoming active during the S phase of the cell cycle (Tachibana et al., 2005). Minichromosome maintenance proteins (MCMs 2-7) are involved in origin activation by binding to or 'marking' DNA for replication. Phosphorylation of these proteins during replication displaces them from the DNA, consequently the DNA is no longer marked ensuring replication is not repeated (Bell and Dutta, 2002).

MCM proteins have been used as biomarkers of cell cycle state as they are highly expressed in all phases of the cell cycle and degraded on cycle exit (Stoeber et al., 2001). Furthermore, in normal stratified epithelium MCM protein expression is only found in the basal proliferative compartment and not in the terminally differentiated keratinocytes. Pre-invasive lesions cause the expansion of the proliferative compartment and resulting neoplastic cells found on the surface of the epithelium are positive for MCM proteins (Freeman et al., 1999). MCM proteins have been found to be highly expressed in a variety of malignant cancers and pre-cancerous cells (Lei, 2005). In this study MCM proteins 2, 4, 5 and 6 were found to be over-expressed in Group 3 tumour cultures compared to normal controls and Group 1 and 2 tumour cultures suggesting disruption of DNA replication mechanisms. Moreover, the over-expression of MCM

protein 2 in meningiomas correlated with an increased risk of recurrence (Hunt et al., 2002), and over-expression in oligodendroglioma correlated with increased tumour grade and poor prognosis (Wharton et al., 2001).

Differential expression of *TP53*, *APC/C* and *MCM* play crucial roles in the disruption of cell cycle regulation and DNA replication in the astrocytomas of this study. It is possible that these alterations lead to increased proliferation, decreased apoptosis and disrupted DNA replication causing chromosome copy number alterations.

Comparison of adult GBMIV and paediatric astrocytoma short-term cell culture expression profiles

The clustering of adult and paediatric astrocytoma short-term cell cultures and the expression profiles of these tumour cultures revealed some unexpected findings. The clustering of 5 of the adult tumour cultures with the 3 paediatric cultures that show alterations of whole chromosomes or arms, suggests that these paediatric tumours may have a genotype more comparable to that of adult astrocytoma than other paediatric astrocytoma. Moreover, the clustering of IN2675 and IN1566 that showed no whole chromosome alterations suggests that the expression profile of these tumours is similar to that of adult astrocytoma but the mechanisms involved in tumour development are different. Furthermore, GBMIV cultures IN2675, IN1566, IN699, IN1523 and IN179 are from paediatric patients that are 14, 6.4, 15, 10 and 13 years of age respectively, suggesting that a sub-group of GBMIV in older paediatric patients are more comparable to adult GBMIV than other paediatric GBMIV patients of a similar age or younger GBMIV patients. The remaining paediatric GBMIV tumours, IN2087 and IN1163, that cluster separately from those previously mentioned are tumours are from patients aged 3 and 15.9 years at diagnosis.

The investigation of genes commonly differentially expressed in adult astrocytoma in the adult and paediatric short-term cell cultures studied here reveal some consistent patterns, but no correlations with tumour grades or short-term cell culture cluster Groups were identified. The integration of the adult and paediatric short-term cell culture samples when completing this clustering approach also suggests that the impact of cell culture may influence gene expression, the majority of which are involved in the cell cycle, promoting integration.

The cluster of 94 gene probe sets that showed over-expression in the sub-group of Group 3 tumour cultures correlated with 53 probe sets that characterised the paediatric short-term cell culture Groups. The remaining 41 probes, representing 38 genes, were only found in the 4870 gene probe set that characterise the Groups when the adult short-term cell cultures are added (Table 5.9).

The function of these 38 up-regulation genes may provide astrocytoma cells with selective advantages. These genes encode proteins that are mostly involved in the cell cycle, proliferation and chromosome instability. Those genes found to be over-expressed that have been shown to promote cell proliferation include *CCNB2*, *CCNB1* and *CDC6*. Furthermore, a large number of genes have functions linked to mitosis, spindle formation and DNA replication including *BUB1B*, *AURKB*, *NUSAP1*, *CENPF*, *RCF4*, *HMMR*, *CKAP2*, *MCM3* and *PTTG1*. Several genes were also associated with chromosome structure including *NASP*, *LBR* and *AURKB*.

Of the remaining genes with known functions *RRM1* has been shown to be involved in chemoresistance, *RAD21* is thought to play a role in DNA repair and *MELK* inhibits specific pro-apoptotic signals. Moreover, the gene coding for the Ki-67 antigen is used to establish the mitotic index of malignancies and up-regulation is associated with more aggressive tumours.

From this analysis it is clear that the tumour cultures of the Group 3 sub-group show additional differential gene expression that can be linked to an aggressive malignant phenotype. As this sub-group of Group 3 consists of adult and paediatric GBMIV short-term cell cultures, the differential gene expression of these genes in paediatric tumours may suggest a tumour that is more similar adult GBMIV.

Genomic CNAs in paediatric astrocytoma short-term cell cultures

In total, 37 clones were found to be altered in 16% of tumour cultures or more. In the majority of cultures these were single clone alterations, but in specific cases clone alterations were part of whole chromosome or arm gains or losses. These included, loss at 6q25.1-6q25.3 in IN2044, loss of 7p21 in IN2012, gain of clones located on chromosome 7 in tumours IN3032, IN2017, IN1523, IN179 and IN699, loss of 2q21-2q22 and 4q13-4q13 in IN1523, loss of 6q25.3 in IN699 and gain of 5p15.2, 9q32-

9q33.3, 1p34.3-1p36.11 and 1p31.1-1p31.3 in IN699. In total, 21 of the 37 clones were found in regions of LCV in the normal population, suggesting that a number of these alterations may either predispose individuals to tumour development or are not involved in tumour development. Of the clones not located in regions of LCV, a large number were located on 7q (25%), a region linked to astrocytoma development.

Of the clones that showed alterations of 2-4 adjacent clones several over-lapped with the single clones. These included a region at 16p11.2 altered in tumour cultures IN3013, IN2591, IN2017 and IN179, a region at 7q11.23 altered in cultures IN1523, IN179 and IN699, a region at 6q12 altered in culture IN2110 and a region at 2q14 altered in culture IN3013.

When investigating the number of individual clones altered in each tumour culture, IN1523 showed the most clone gains and losses (1031). However, IN699 does show the most chromosome alterations. This is in part due to IN1523 showing several alterations of whole chromosomes compared to regions in IN699, but also the number of clones that were accepted as reliable during experimental analysis. The aCGH experiment investigating tumour culture IN699 had a high number of clones, 34%, that were deemed unreliable. This ranged from 2-45% in the remaining cultures investigated and explains the large difference in single clones altered in each culture case (refer to the Supplementary Data CD for clones deemed unreliable for each individual aCGH experiment).

Copy number alterations and astrocytoma predisposition

Specific germline gene mutations have long been associated with an increased risk or predisposition to disease including *TP53* mutations and the risk of Li-Fraumeni Syndrome and *BRAC1* mutations in breast cancer (Frebourg, 1997a; Gilmore et al., 2003). The characterisation of frequent LCVs involving gain or loss of hundreds of kilobases of genomic DNA in phenotypically normal individuals has suggested that some regions of LCV maybe involved in disease predisposition (Iafrate et al., 2004). A recent study that investigated the risk of cancer in 2561 patient with constitutional chromosome deletions found that an increased risk of renal cancer was seen in patients with 11p loss, an increased risk of eye cancer was found in patients with loss of 13q and

loss of 11q increased the risk of vulval and vaginal cancer and anogenital cancers (Frebourg, 1997b; Gilmore et al., 2003; Swerdlow et al., 2008).

Few studies have investigated germline genomic alterations in adult or paediatric astrocytoma patients. Diaz de Stahl et al. (2005) identified a small gain on chromosome 22q in both germline and adult GBMIV DNA in 28% of patients investigated. This region included the genes *TOP3B*, involved in DNA replication, and *TAFA5*. No CNA in this region were identified in the tumours of this study. The study by Braude et al. (2006) concluded the CNA at 14q12 present in 50% of chronic myeloid leukaemia (CML) and paediatric solid tumours compared to an incidence of 10% in somatic DNA of healthy controls suggested that DNA alterations at this site may predispose individuals to tumour development. This region needs further investigation in paediatric astrocytoma as discussed in this Chapter and Chapter 4. Furthermore, a study investigating cancer incidence in women with Turner syndrome found that the risk of CNS tumours, specifically meningioma and childhood brain tumours, was significantly increased in this patient cohort. Moreover, the patient in this study with tumour IN1419 had been diagnosed with Turner syndrome and showed no other CNAs aside from loss of 1 copy of chromosome X. Gain of 7q was the most frequent large CNA found in the short-term cultures of this study but has not previously been associated with predisposition to astrocytoma. However, this alteration has been linked to tumour development and progression as discussed later in this Chapter.

Somatic mutations in cancer candidate genes have also been identified in patients with GBMIV including *TP53* located at 17p13.1, *NF1* located at 17q11.2, *EPHA3* located at 3p11.2, MLL3 located at 7q36.1, *TECTA* located 11q22-q24, FBXW7 located at 4q31.3, and *OBSCN* located at 1q42.13 (Thiel et al., 1995; Fulci et al., 2002; Balakrishnan et al., 2007). No alterations in these regions were identified in the tumours of this study. However, larger regions encompassing these loci, most likely to be involved in tumour development and/or progression rather than predisposition, were found including 17p13.1 gained in IN699, 7q36.1 gained in 4 tumours including IN2017, IN3032, IN179 and IN1523, chromosome 4 was lost in IN1523 and IN699 and 1q32-1pter was lost in IN699. Any of the 21 loci identified by single clone alterations in this study and located in regions of LCV common in the normal population, could be

investigated further, to establish if these are germline alterations and may predispose individuals to tumour development.

Correlation of CNAs with differential gene expression in paediatric astrocytoma short-term cell cultures

Reliable expression data was not available for the majority of single clone alterations investigated. The most frequently altered clone was located at 1p13.3-1p21.1 and a cluster of amylase genes, including α-amylase, mapped to the clone location. No reliable expression data was available for these genes, but reduced expression of α-amylase has been found in AAIII and GBMIV compared to normal brain (Kotonski et al., 2001). The gene *COL11A1* is adjacent to the clone at 1p13.3-1p21.1 and was found to be under-expressed in all astrocytoma cultures. Down-regulation can be explained by clone loss at 1p13.3-1p21.1 in 60% of cultures. An additional mechanism such as promoter hypermethylation is likely to be involved in the down-regulation of *COL11A1* in the remaining 40% of tumour cultures.

CNAs were seen in 47% of cultures at 14q12 and were randomly distributed between the 3 tumour culture Groups. In total, 28% of cultures showed gain and 19% showed loss, compared to 82% of PAI biopsies that displayed loss in Chapter 4. As previously discussed, 14q12 is a region of LCV in the normal population and alterations at this site have been associated with solid tumours in children (Braude et al., 2006). A cluster of genes including *RPL26P*, *BTF3P3* and *LOC387978* map to the exact clone location at 14q12, but no reliable expression data was available. The gene *FOXG1B* is adjacent to the clone at 14q12 and was found to be under-expressed in all PAI biopsies investigated in Chapter 4. Moreover, *FOXG1B* expression was shown to be down-regulated in 91% of the paediatric astrocytoma cultures studied here; IN699, IN1523 and IN179 showed up-regulation. A recent study identified loss at 14q12 in 75% of hepatoblastoma but over-expression of *FOXG1B* in 81% of cases. This was suggested to be the result of errors in duplication/deletion in adjacent regions at 14q12 (Adesina et al., 2007). CNAs at this site and the involvement of *FOXG1B* in paediatric astrocytoma require further investigation.

A clone located a 10q11.22 was found to be lost in ten tumours that were randomly distributed between the 3 tumour culture Groups. This correlated with down-regulation

of *ANXA8* in all tumours. This gene is a calcium-dependent phospholipid-binding protein previously identified as a blood anticoagulant (Hauptmann et al., 1989). Up-regulation of *ANXA8* has been found in acute promyelocytic leukaemia and breast cancer as a marker or poor prognosis (Sarkar et al., 1994; Stein et al., 2005).

The up-regulation of *EPHA2* located at 1p35.1-p36.21 has been associated with aggressive malignancies and tumour progression (Fang et al., 2005). This is contradictory to this study, which found under-expression of this gene seen in all tumour cultures compared to normal controls and clone loss at 1p35.1-p36.21 in 8 cultures. Furthermore, the up-regulation of ephrin-A1 in U251 adult GBMIV cells and consequent down-regulation of *EPHA2* inhibits migration, cell proliferation, and adhesion-independent growth highlighting a key role for *EPHA2* in tumourigenesis (Liu et al., 2007). This suggests that while the down-regulation of *EPHA2* may inhibit tumourigenesis in adult astrocytoma, the role of this gene in paediatric astrocytoma needs further investigation. It may be that the down-regulation of *EPHA2* in low-grade astrocytoma does inhibit tumour growth but that in high-grade astrocytomas alternative mechanisms promote tumour development.

TJP1 located at 15q13.1 was found to be over-expressed in Group 2 and 3 tumour cultures and correlated with gain of clone AC102941.9 in 7 cultures. In contrast to the expression profile, 5 of the 7 cultures that showed gain of clone AC102941.9 cluster in Group 1. This gene encodes a protein located on the cytoplasmic membrane surface of intercellular tight junctions and is involved in signal transduction at cell-cell junctions. Altered expression of *TJP1* has been associated with the differentiation of liver metastases and colorectal cancer. Furthermore, the up-regulation of this gene has been shown to contribute to melanoma ontogenesis (Kaihara et al., 2003; Smalley et al., 2005).

ARFHGEF4 was down-regulated in all paediatric astrocytoma cultures and is located at 2q21-q22, a region found to be lost in 5 tumour cultures. The protein of this gene (also known as APC stimulated guanine nucleotide exchange factor (Asef)), is a key regulator of APC and actin cytoskeleton reorganisation. Mutant APC binds Asef constitutively activating the protein and increasing cellular migration (Kawasaki et al., 2003). Furthermore, adenoma cells of APC knock-out mice have a similar proliferation rate to

normal crypt epithelium, but the cells lack direction suggesting that aberrant migratory behaviour may be important for tumour formation as well as progression and invasion (Oshima et al., 1997; Akiyama and Kawasaki, 2006). The evidence does suggest that the down-regulation of *Asef* may contribute to paediatric astrocytoma development through disruption of cytoskeleton organisation.

COL8A2, located at 1p34.3-1p36.11, was up-regulated in Group 2 tumour cultures, *G10 (BUD31)*, located at 7q21.1-7q21.1, was up-regulated in Group 2 and 3 tumour cultures and *SHCBP1,* located at 16p11.1-16p11.1, was up-regulated in Group 3 cultures. Roles for these genes in tumour development have not been characterised. However, *COL8A1* from the same protein family as *COL8A2* was found to be up-regulated in gastrointestinal stromal tumours and has been linked to malignant tumours with an increased risk of recurrence (Koon et al., 2004).

TRIP6, located at 7q21.1-7q21.1, was up-regulated in Group 2 and 3 tumour cultures. The protein of this gene functions as a coactivator for AP-1 and NF-kB, acting as a molecular platform for transcription factors (Kassel et al., 2004). This is a key signalling cellular pathway often disrupted in tumour development, suggesting a role for this gene in tumourigenesis.

ARPC1A, located at 7q21.1-7q21.1, was up-regulated in Group 3 tumour cultures. The protein encoded by this gene is a subunit of the Arp2/3 complex. This complex is involved in the organisation of the actin cytoskeleton. Moreover, this complex was found to be enriched in the lamellipodia of fibroblasts on stimulation with PDGF, suggesting that this protein may be involved in cell motility (Machesky et al., 1997).

The genes *SGCE* and *SLC25A13* were up-regulated in all paediatric astrocytoma cultures and are located at 7q22.1, which showed gain in 5 tumour cultures. Mutations in *SLC25A13* have been characterised in adult-onset citrullinemia (type II, CTLN2), a disease associated with a high incidence of hepatocellular carcinoma (Tsai et al., 2006). This suggests that disruption of this gene may be involved in tumourigenesis. The *SGCE* gene encodes the epsilon member of the sarcoglycan family of proteins and is highly expressed in the brain (Nishiyama et al., 2004). A role for this gene in tumour development remains to be determined.

Although no reliable expression data was available for the genes located at 19p13.2, this region has been identified as a cancer susceptibility locus in breast cancer. The over-expression of ICAM1 in this region strongly correlates with breast tumourigenesis (Rosette et al., 2005). Moreover, 5 tumour cultures showed gain of a single clone at 19p13.2. Other regions of CNAs that could not be correlated with differential gene expression have shown alterations in other types of cancer including 16p11.2 in small cell lung carcinoma and breast cancer, 19p13.2 in ependymoma, 7p21 in ependymoma and prostate cancer, and 7q11.23 in prostate cancer and cholangiocarcinoma (Levin et al., 1994; Ichikawa et al., 2000; Chariyalertsak et al., 2005; Modena et al., 2006; Stange et al., 2006). Furthermore, loss of a region at 7q11.23 was also found 7 PAI biopsies investigated in Chapter 4

Individual tumour cultures showed alterations of multiple adjacent clones ranging from 2 to 218 (Table 5.12). The investigation of these clones in individual cultures showed correlation with differential gene expression in the same regions. Of the differentially expressed genes that correlated with adjacent clone alterations in specific cultures, 5, including *ARFHGEF4*, have functions that may promote tumour development or provide the specific tumour with a selective advantage. *CASP6* is a member of the cysteine-aspartic acid protease (caspase) family. Caspase activation plays a central role in the initiation of cell apoptosis, consequently the down-regulation of this gene in tumour IN2122 may inhibit apoptosis. Reduced *CASP6* expression has been found in approximately 50% of gastric cancers, as well as mutations, although at a low frequency (Yoo et al., 2004; Lee et al., 2006). Caspase-8, -3 and -6 have also been shown to directly proteolyse β-catenin *in vitro* (Van de Craen et al., 1999).

Previously, 4q32-34 has been characterised as a susceptibility locus for familial pancreatic cancer. *PALLD,* a gene located in the region that encodes a component of the cytoskeleton that controls cell shape and motility, was found to be mutated in all affected family members but not the unaffected members. Furthermore, over-expression of *PALLD* was reported in the tissues from precancerous dysplasia and pancreatic adenocarcinoma in both familial and sporadic disease. Transfection of wild-type and mutant *PALLD* gene constructs into HeLa cells, initiated several phenotypic effects. The cells expressing the mutant construct exhibited cytoskeletal changes, abnormal actin bundle assembly and an increased migration. This suggests that *PALLD* over-expression

may contribute to cell invasion and migration in tumour IN2110 (Pogue-Geile et al., 2006).

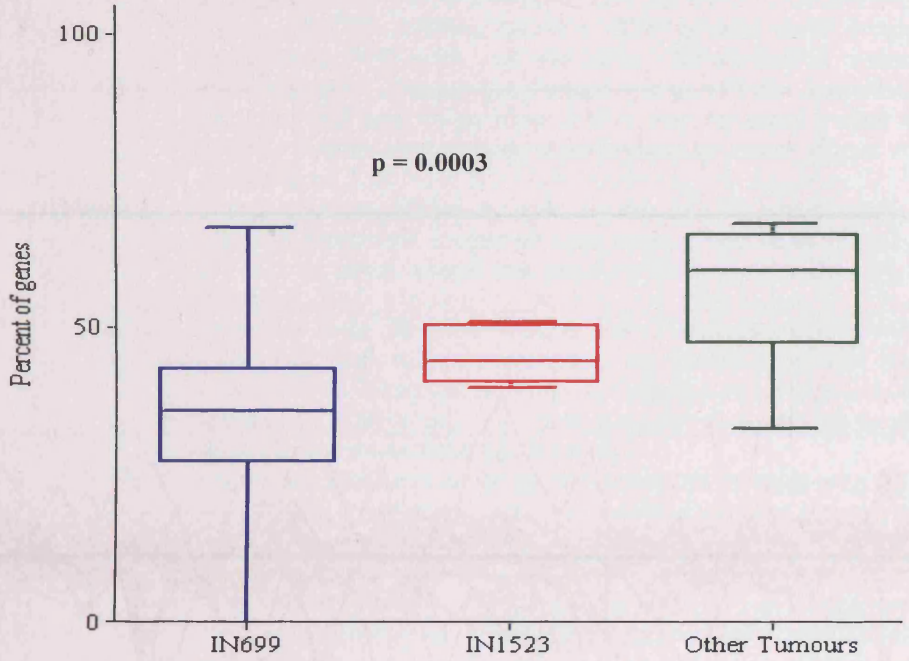
PDIA2 is specifically up-regulated in response to hypoxia/brain ischemia in astrocytes. Furthermore, the over-expression of this gene in neurons protects against apoptotic cell death induced by hypoxia/brain ischemia. The up-regulation of *PDIA2* in neuronal cells significantly inhibited hypoxia-triggered DNA fragmentation. This was not seen when the DNA fragmentation was induced by nitric oxide or staurosporine. This suggests that *PDIA2* is a crucial regulatory protein involved in cell death and therefore up-regulation may result in tolerance against ischemic stress in tumour IN178 (Ko et al., 2002).

Multidrug resistance can be caused by ATP-binding cassette (ABC) transporters that function as drug efflux pumps. The over-expression of ABCA3 was found in 42 samples from children with acute myloid leukaemia. Furthermore, the median expression of ABCA3 was higher in 21 patients who did not achieve remission after the first course of chemotherapy. Incubation of leukaemia cell lines with a number of different cytostatic drugs increased ABCA3 expression. Moreover, the down-regulation of ABCA3 by small interfering RNA sensitized cells to specific chemotherapeutic agents (Steinbach et al., 2006). This suggests that the up-regulation of this gene in tumour IN178 may increase drug resistance in this tumour.

In all tumor cultures except IN699, 40-68% of differentially expressed genes that correlated with CNAs involving more than 2 clones were involved in the profile of the 4682 probe sets used to characterise the clustered tumour culture Groups. In culture IN699 this was reduced to 0-55%. Figure 5.50 illustrates a significant difference between the mean percentage of differentially expressed genes located in regions of alteration and also involved in the profile of the 4682 probe sets for culture IN699, culture IN1523 and the regions altered in the remaining cultures that were larger than 4 adjacent clones. Regions of alteration involving 4 clones or less were not included in this approach as only a small number of genes in these regions showed differential gene expression. A significant difference in the mean number of these 3 Groups was established using a 1-WAY-ANOVA ($p = 0.0003$). This suggests that CNAs are influencing differential gene expression involved in a common tumorigenic profile as well as additional genes that may provide individual tumours with selective advantages,

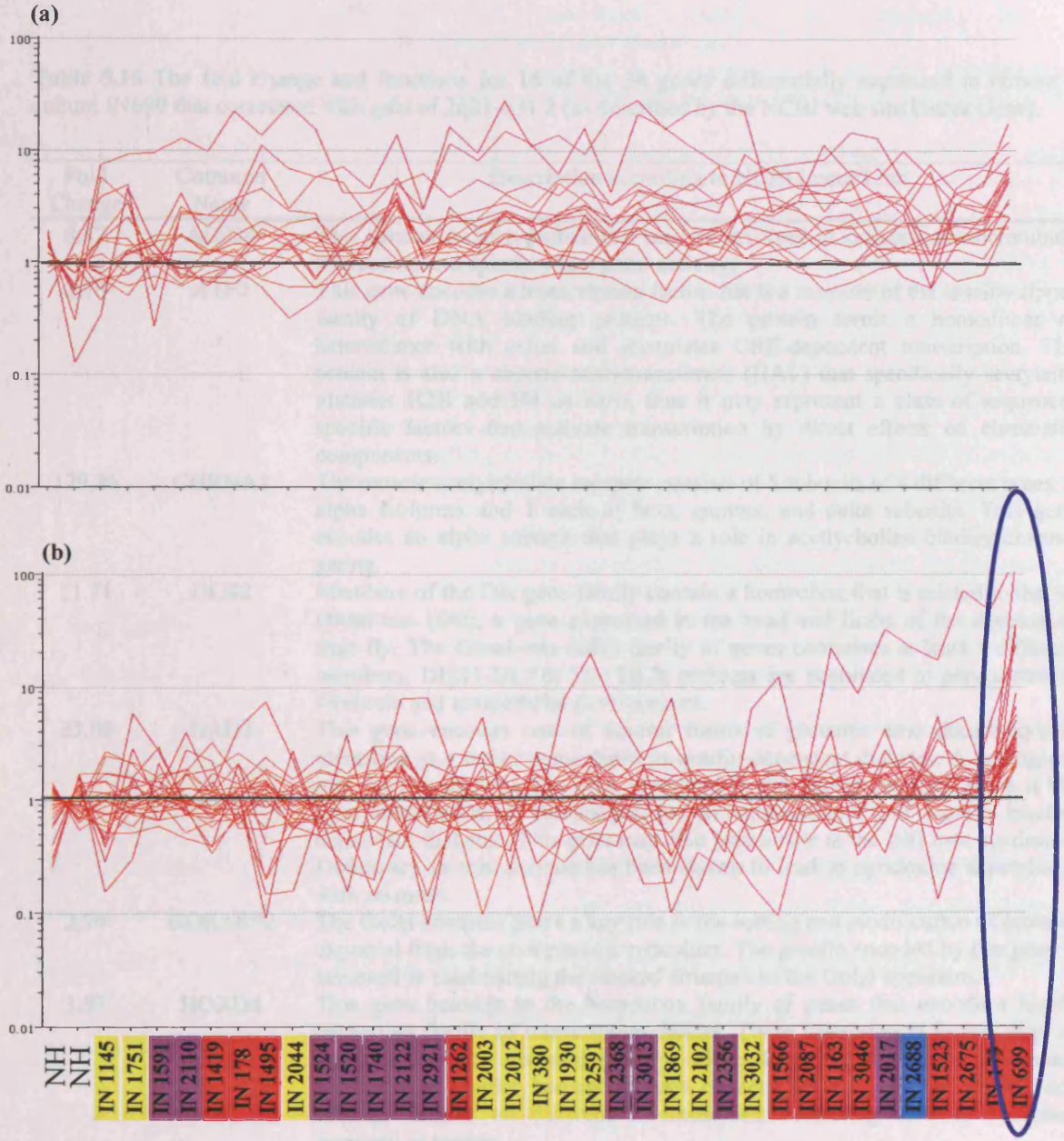
promoting tumour development. The lower percentage of differentially expressed genes in culture IN699 that correlate with the common tumorigenic profile suggests an increase in the number of differentially genes that are specific to this tumour and may provide a selective advantage.

Figure 5.50 This Figure illustrates the significant difference between the mean percentages of differentially expressed located in regions of CNA and involved in the profile of the 4682 probe sets for tumour IN699, tumour IN1523 and other tumours.



As an example of relationship between CNAs and differentially expressed genes common to the tumorigenic profile and those specific to an individual tumour, the expression profile of 53 differentially expressed genes that correlated with gain of 2q21-q31.2 in IN699 is illustrated in Figure 5.51. The top profile shows 19 genes that correlate with the 4682 differentially expressed probe sets used to characterise the culture clusters. The bottom profile illustrates the 34 differentially expressed genes in IN699 only (the genes showed a 2-fold up-regulation in IN699 compared to the remaining tumours). The gene expression profile of the 34 genes shows a constant expression in all tumours (variations in specific tumours can be seen) and up-regulation in IN699, compared to the profile of the 19 genes that shows a continuous increase in expression from Group 1 to 3 tumour cultures.

Figure 5.51 The expression profile of 53 differentially expressed genes that correlated with gain of 2q21-q31.2 in IN699.



The top profile (a) illustrates the 19 genes also found in the 4682 genes characterising the tumour clusters. The bottom profile (b) illustrates the 34 differentially expressed genes in IN699.

To establish whether or not the genes up-regulated in IN699 may provide the tumour with a selective advantage, the functions of 34 genes showing up-regulation in IN699 only were studied in detail. Table 5.16 illustrates the fold change and functions as described by the National Centre for Biotechnology Information (NCBI) web site Entrez Gene (www.ncbi.nlm.nih.gov/entrez) of 16 characterised genes from the 34 that showed a 2-fold up-regulation in tumour culture IN699 only. The functions of these genes are linked to cellular processes and mechanisms that if disrupted could promote

tumour development. Furthermore, several of these genes have already been associated with various cancers.

Table 5.16 The fold change and functions for 16 of the 34 genes differentially expressed in tumour culture IN699 that correlated with gain of 2q21-q31.2 (as described by the NCBI web site Entrez Gene).

Fold Change	Common Name	Description according to NCBI Locus Link
8.47	AD024	This gene encodes a protein that may be involved in kinetochore-microtubule interaction and spindle checkpoint activity.
2.76	ATF2	This gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. The protein forms a homodimer or heterodimer with c-Jun and stimulates CRE-dependent transcription. The protein is also a histone acetyltransferase (HAT) that specifically acetylates histones H2B and H4 *in vitro*; thus it may represent a class of sequence-specific factors that activate transcription by direct effects on chromatin components.
179.86	CHRNA1	The muscle acetylcholine receptor consists of 5 subunits of 4 different types: 2 alpha isoforms and 1 each of beta, gamma, and delta subunits. This gene encodes an alpha subunit that plays a role in acetlycholine binding/channel gating.
11.71	DLX2	Members of the Dlx gene family contain a homeobox that is related to that of Distal-less (Dll), a gene expressed in the head and limbs of the developing fruit fly. The Distal-less (Dlx) family of genes comprises at least 6 different members, DLX1-DLX6. The DLX proteins are postulated to play a role in forebrain and craniofacial development.
33.00	GAD1	This gene encodes one of several forms of glutamic acid decarboxylase, identified as a major autoantigen in insulin-dependent diabetes. A pathogenic role for this enzyme has been identified in the human pancreas since it has been identified as an autoantigen and an autoreactive T cell target in insulin-dependent diabetes. This gene may also play a role in the stiff man syndrome. Deficiency in this enzyme has been shown to lead to pyridoxine dependency with seizures.
2.79	GORASP2	The Golgi complex plays a key role in the sorting and modification of proteins exported from the endoplasmic reticulum. The protein encoded by this gene is involved in establishing the stacked structure of the Golgi apparatus.
3.57	HOXD4	This gene belongs to the homeobox family of genes that encode a highly conserved family of transcription factors. These transcription factors play an important role in morphogenesis in all multicellular organisms. Mammals possess four similar homeobox gene clusters, HOXA, HOXB, HOXC and HOXD, located on different chromosomes, consisting of 9 to 11 genes arranged in tandem.
2.78	HOXD9	As HOXD4
147.93	ITGA6	The ITGA6 protein product is the integrin alpha chain alpha 6. Integrins are integral cell-surface proteins composed of an alpha chain and a beta chain which are known to participate in cell adhesion as well as cell-surface mediated signalling.
2.28	MTX2	The protein encoded by this gene is highly similar to the metaxin 2 protein from mouse, which has been shown to interact with the mitochondrial membrane protein metaxin 1. Because of this similarity, it is thought that the encoded protein is peripherally associated with the cytosolic face of the outer mitochondrial membrane and be involved in the import of proteins into the mitochondrion.

3.89	PDK1	Pyruvate dehydrogenase (PDH) is a mitochondrial multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate and is one of the major enzymes responsible for the regulation of homeostasis of carbohydrate fuels in mammals. The enzymatic activity is regulated by a phosphorylation/dephosphorylation cycle.
2.85	POH1	POH1 (pad one homolog-1) is a component of the 26S proteasome, a multiprotein complex that degrades proteins targeted for destruction by the ubiquitin pathway.
4.03	RBMS1	This gene encodes a member of a small family of proteins which bind single stranded DNA/RNA. The proteins of this family have been implicated in the diverse functions of DNA replication, gene transcription, cell cycle progression and apoptosis. Several isforms of this gene were isolated by virtue of their binding to either strand of an upstream element of c-myc (MSSPs), or by phenotypic complementation of cdc2 and cdc13 mutants of yeast (scr2), or as a potential human repressor of HIV-1 and ILR-2 alpha promoter transcription (YC1).
2.07	SSB	La is involved in diverse aspects of RNA metabolism, including binding and protecting 3-prime UUU(OH) elements of newly RNA polymerase III-transcribed RNA, processing 5-prime and 3-prime ends of pre-tRNA precursors, acting as an RNA chaperone, and binding viral RNAs associated with hepatitis C virus.
3.03	TANK	The TRAF (tumor necrosis factor receptor-associated factor) family of proteins associate with and transduce signals from members of the tumor necrosis factor receptor superfamily. The protein encoded by this gene is found in the cytoplasm and can bind to TRAF1, TRAF2, or TRAF3, thereby inhibiting TRAF function by sequestering the TRAFs in a latent state in the cytoplasm. For example, the protein encoded by this gene can block TRAF2 binding to LMP1, the Epstein-Barr virus transforming protein, and inhibit LMP1-mediated NF-kappa-B activation.
2.96	WASPIP	This gene encodes a protein that plays an important role in the organization of the actin cytoskeleton.

The involvement of *ATF2* in MAPK signalling and tumour cell invasion has previously been investigated in breast tumour and GBMIV cells (Song et al., 2006; Dziembowska et al., 2007). A central role for the cholinergic receptor, nicotinic, alpha 1 (muscle) (CHRNA1) in the regulation of growth factor-induced endothelial cell migration provides evidence that this gene is involved in tumour development (Ng et al., 2007). The differential gene expression of *HOXD4* and *HOXD9* has been associated with acute lymphoid malignancies and esophageal squamous cell carcinomas respectively. The up-regulation of *ITGA6* may be involved in increased Akt signalling in breast cancer (Shen and Falzon, 2006).

The investigation of differential gene expression that correlated with gain of 2q21-q31.2 in tumour IN699 provided evidence that CNAs may influence differential gene expression of genes involved in a common tumour profile as well as specific genes in individual tumours. Moreover, it has been shown that differentially expressed genes in a

region of CNA in one tumour are also part of a common tumourigenesis profile in other tumours that do not show CNA in the same region.

Gain of part or all of chromosome 7 and correlation with gene expression in paediatric astrocytoma short-term cell cultures

Gain of part or all of chromosome 7 was the most common chromosome alteration found in the paediatric astrocytoma short-term cell cultures studied here. Furthermore, all 5 cultures with this CNA clustered in Group 3 including the PAI, IN2017. Of the 5 patients with tumours showing chromosome 7 alterations, four died due to their tumour and 1 remains alive (IN2017).

In total, 16 genes located on chromosome 7 showed a 2-fold and significant up-regulation in expression in tumours with gain of part or all of chromosome 7. Furthermore, all of these genes correlate with the 4682 probe sets that were used to characterise the tumour culture clusters. The profile of these genes in Figure 5.42 shows a continuous increase in expression between Group 1 and 3 tumour cultures. Moreover, 5 of these genes have functions previously linked to cancer or tumour development including astrocytoma.

The over-expression of calcium binding protein *SRI* has recently been described as a marker of GBMIV (Yokota et al., 2006) Furthermore, the over-expression of the SRI protein in multi-drug resistant (MDR) leukaemia cells lines led to reduced cytosolic calcium levels and increased resistance to apoptosis through the up-regulation of *Bcl-2* and decreased level of *Bax*. This suggests that SRI plays a role in MDR via the regulation of apoptosis pathways (Qi et al., 2006).

PBEF1 encodes a protein that is involved in the biosynthesis of nicotinamide adenine dinucleotides. In addition, to functions promoting vascular smooth muscle cell maturation and inhibition of neutrophil apoptosis, PBEF1 has been shown to promote angiogenesis via activation of mitogen-activated protein kinase ERK-dependent pathway in adypocytes (Kim et al., 2007). Furthermore, this protein has been shown to activate migration, invasion and tube formation in human umbilical vein endothelial cells (Kim et al., 2007). Differential expression of *PBEF1* was also shown between drug resistant and sensitive blasts in acute myeloid leukaemia (Eisele et al., 2007).

The protein encoded by *CAPZA2* is the alpha subunit of the barbed-end actin binding protein Cap Z. By capping the barbed end of actin filaments, Cap Z regulates the growth of the actin filaments. Furthermore, this gene is located in a gene cluster found to be amplified on chromosome 7 in more than 20% of GBMIV investigated (Mueller et al., 1997).

The protein encoded by the *ABCF2* gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intracellular membranes. Copy number gains of this gene have been associated with a drug resistant phenotype in a variety of cancers (Yasui et al., 2004). Up-regulation of *ABCF2* has also been found in clear cell adenocarcinomas of the ovary and the uterine corpus (Nishimura et al., 2007).

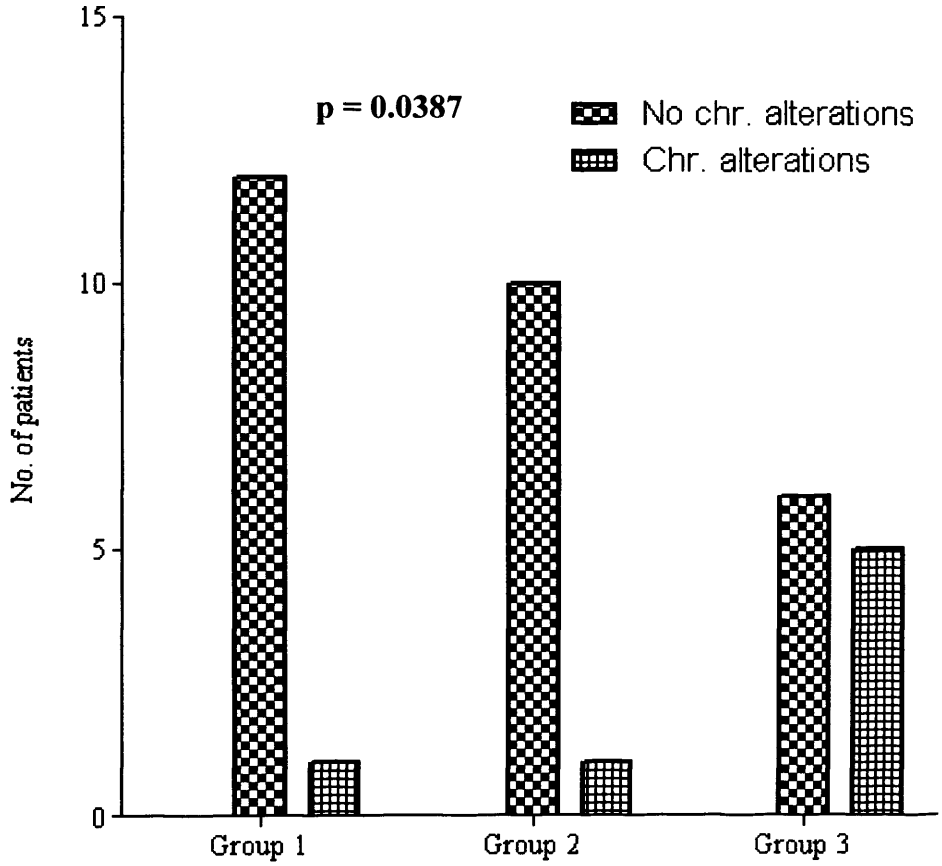
Gain of chromosome 7 has previously been associated with reduced survival in adult DAII, AAIII and GBMIV (Kunwar et al., 2001; Wessels et al., 2002; Arslantas et al., 2004). It has been suggested that the gain of chromosome 7 accumulates with age in GBMIV (Zuber et al., 2002) and gain of 7q has been proposed as an early event in the initiation of adult astrocytoma (Schrock et al., 1996). Furthermore, radiation resistant adult GBMIV cells showed gain of 3 regions on chromosome 7 (Misra et al., 2006). Part of one of these regions (7q21.1-7q22.1) showed gain in all 5 of the paediatric astrocytoma short-term cell cultures with alterations on chromosome 7 studied here, suggesting that resistance to radiation may also be linked to up-regulation of genes on chromosome 7.

In this tumour cohort, 5 patients with tumour cultures showing gain of part or all of chromosome 7 and 2 patients with tumours that showed alterations of 6q and 7p were 7 years of age or above, suggesting that whole chromosome or arm alterations may be more common in tumours of older children. However, this was not found to be significant when comparing the number of children above 7 years of age with tumour cultures that showed whole chromosome or arm alterations (two-sided Fisher's Exact Test $p = 0.0721$).

Further discussion of paediatric astrocytoma short-term cell culture Groups 1, 2 and 3

The tumour cultures with multiple whole chromosome or arm alterations all clustered in Group 3. Figure 5.53 illustrates the significant difference between the number of tumour cultures with whole chromosome or arm alterations in Group 3 compared to Groups 1 and 2 and this is independent of tumour grade (Chi-square test p = 0.0387). However, this may be due to the small number of tumours with whole chromosome alterations and the small number of AAIII in this study.

Figure 5.53 This graph illustrates the significant increase in tumours with whole chromosome or arm alterations in Group 3



In this study, paediatric astrocytoma cultures cluster in 3 clear Groups, each with a specific expression profile. The profiles of Group 1 and 2 tumour cultures appear to be characteristic of predominantly low-grade tumours with the profile of Group 3 tumour cultures being associated with predominantly high-grade tumours. The expression profile is a reflection of CNAs, in that the majority of tumour cultures with multiple

whole chromosome or arm alterations cluster in Group 3, suggesting that gene dosage of multiple genes is influenced by CNAs in these tumours. The expression profile of the remaining tumour cultures in Group 3 that show only a small number of single alterations, may be due to alternative mechanisms other than CNAs or CNAs that are smaller than 1MB and undetectable by aCGH. Tumour short-term cultures that cluster in unexpected groups can be explained to a certain extent by specific tumour characteristics, including the GA variant of tumour IN3032; the single clone CNAs found in IN1495, IN1419, IN178 and IN1262 and the gain of chromosome 7q in tumour IN2017.

The clustering of these tumour short-term cultures predominately by grade does suggest that these cultures are representative of the tumour of origin with some of high-grade cultures showing large and more frequent CNAs. As discussed in the introduction the majority of paediatric astrocytomas previously investigated (>70%) have a normal karyotype (Bhattacharjee et al., 1997), therefore only small number of tumours were expected have abnormal complex karyotypes. However, as the short-term cultures were derived from tumour biopsy tissue, this approach may have introduced non-tumorigenic cells into the culture or generated a mixed clonal population as discussed in Chapter 3. This may also explain the expression profiles and clustering of tumour cultures IN1495, IN1419, IN178 and IN1262. However, given that previous cytogenetic studies have only identified a small number of abnormal karyotypes in paediatric astrocytoma and the differential expression profiles of all tumour cultures here, this is unlikely and it is probable that alternative mechanisms are influencing gene expression or that CNAs smaller the 1KB are present in these short-term tumour cultures and undetectable by the approach used here.

It is also important to recognise that the tumour cluster Groups may be a representation of tumour heterogeneity. This study involved short-term cell cultures of passage 8 or less and consequently the expression profile, although influenced by short-term cell culture, has not undergone the changes associated with long-term culture or that of cell lines. The profiles may not be representative of the majority clone within each tumour and it is also possible that less common clones capable of growing in an *in vitro* environment were grown during culture. Moreover, it is also feasible that the tumour Group expression profiles are a reflection of tumour region representing the tumour

periphery or a more central area of the tumour mass. These are two scenarios outside the scope of this study as it was not possible to know exactly which region of the tumour mass a sample was taken from or the exact clonal composition of the tumour sample.

In conclusion the author feels that the paediatric astrocytomas short-term cell cultures used in this study are representative of the tumour of origin as shown by the clustering of the cultures into 3 Groups, Groups 1 and 2 consisting of predominantly low grade tumours and Group 3 high-grade tumours. This is also strengthened by the increased number of CNA found in the cultures of Group 3, specifically the gain of chromosome 7. The significantly disrupted profiles of each Group and to a greater extent in Group 3, has allowed the identification and characterisation of pathways and genes that maybe involved in tumour development in paediatric astrocytoma.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Paediatric astrocytoma are the most common form of solid tumour in children and are classified by WHO guidelines into 4 grades of malignancy. PAI are most common in patients below 15 years of age, with the majority being located in the cerebellum. The 10-year survival rate of children diagnosed with astrocytoma is approximately 90% for patients with grade I tumours, compared to only 15% for patients with malignant tumours. Current treatment regimes are not adequate for those patients with high-grade tumours and new more effective approaches are needed.

Little is known about the genetics of paediatric astrocytoma compared to their adult counterparts. This study has involved the characterisation of differential gene expression in paediatric astrocytoma, to further understand the genetics involved in tumour development. Clustering of paediatric PAI biopsies, according to expression profile similarity, showed that these tumours are significantly different from normal brain. Furthermore, clustering of paediatric astrocytoma short-term cell culture samples of various grades demonstrated that paediatric astrocytoma could be grouped into low and high-grade tumours by molecular signature. The tumours clustered into 3 clear Groups with Groups 1 and 2 consisting of predominately low-grade tumours and Group 3 comprising mostly high-grade tumours. Furthermore, the tumours of Group 3 were also associated with increased CNAs, specifically gain of part or all of chromosome 7q and this was independent of tumour grade. However, only 20% of tumours showed whole chromosome or arms CNAs and this may have influenced this result, as well as the small number of AAIII.

The assessment of tumour biopsies and short-term cell cultures identified a large number of differentially expressed genes, which were further characterised according to GOs and cellular pathways. Pathways including the cell cycle, Wnt signalling, MAPK signalling and PI3K signalling were shown to be disrupted in paediatric astrocytoma. Key genes involved in these pathways and the role they could play in tumour development was also defined.

Unsupervised hierarchical clustering of paediatric and adult short-term cell cultures revealed surprising results. A cluster of paediatric GBMIV integrated with the adult GBMIV, suggesting that these paediatric tumours may be more similar to their adult counter parts compared to other paediatric astrocytoma. Investigation of the expression profile identified a number of differentially expressed genes unique to this paediatric and adult astrocytoma cluster, which may be used to distinguish paediatric astrocytoma with a similar molecular profile to adult astrocytoma.

The most challenging part of this study was the use of both biopsy and short-term cell culture samples in the investigation of paediatric astrocytoma. It is the majority opinion that tumour tissue is the preferred material in the analysis of any tumour or cancer, however many functional studies require short-term cell cultures and cell lines. It is therefore important that the differences and similarities of tumour tissue and derived cells in culture are clear and understood. Cell culture does have an overall effect on gene expression without a doubt. However, short-term cell cultures do have a molecular profile that represents the tumour of origin. The results in this study using paediatric astrocytoma short-term cell cultures demonstrate that astrocytoma cells derived from tumours of various grades can generally be distinguished. The impact of *in vitro* cell culture influences all derived cells and therefore the molecular profiles from specific grades maintain a relationship similar to that expected when using astrocytoma biopsies. This is an over simplification to a certain extent, as tumour heterogeneity and the isolation of specific cell sub-populations during culture do impact the molecular profile. This study also highlighted that cell culture conditions influence adult GBMIV derived short-term cell culture expression profiles to a lesser extent than paediatric PAI short-term cell culture profiles, but that the derived short-term cell cultures could be distinguished from the other by molecular profile.

The impact of cell culture on gene expression also means that biopsy and short-term cell cultures cannot be combined in the same experiment as biological or technical replicates of paediatric astrocytoma. The tumour samples can only be investigated and compared to other samples of the same source and controls unique to each sample source must be used. Furthermore, direct comparisons between the biopsy and derived short-term cell cultures from the same tumour are not possible, due to the impact of cell culture on gene expression. Moreover, because of the variation and difficulties encountered with

controls and experiment analysis approaches in this study, comparisons between the differentially expressed genes identified in the biopsy and short-term cell culture experiments were felt to be inappropriate. This does not mean to say that any one experiment is better than the other, but that comparisons between the two are not feasible. It would be extremely valuable to carry out a parallel study generating expression profiles of both paediatric astrocytoma tissues and derived cell lines. This would allow more appropriate comparisons to be made between paediatric astrocytoma biopsy and short-term cell cultures of various grades.

Several aspects of this study have demanded careful attention and thought. The use of specific control samples in microarray experiments is usually dictated by the availability and access to controls sources. Appropriate controls should be investigated for each tumour or cancer type. However, lack of published studies in this area hinders control choices. The use of uniform standard controls for studies involving specific tumours or cancer would allow more comparisons to be made between studies. The controls chosen for the analysis of paediatric astrocytoma biopsies and short-term cell cultures in this study have dictated experimental design and to some extent the differentially expressed genes identified in paediatric astrocytoma. The use of controls in the identification of differently expressed genes, between so called 'normal' and 'tumour' samples has a direct influence on the generated gene list, as shown by Zorn et al. (2003). All possible efforts were made in this study to obtain the best controls and where doubts were cast over controls, experimental analysis was adjusted.

The use of adult normal total brain as a control for PAI biopsy analysis was done so with some confidence. The controls showed reduced gene expression variability, associated with individuals, due to pooling and originated from young adults. Comparisons and justifications were carried out for the use of this control, with the only better alternatives being normal brain from a child or from the tumour patient. Two options which realistically are not possible. It has been suggested that astrocytes would be a suitable control for this type of analysis. However, with this approach the control would consist of a uniform cell population, a feature that is uncommon in any tumour.

As the pooled adult normal total brain control was compared to PAI biopsies that clustered tightly together and no other biopsies were available from other astrocytoma

grades, it was felt appropriate to normalise the experiment to the control samples. This approach clearly highlights those genes that are differentially expressed compared to the controls.

It was felt to be inappropriate to compare astrocytoma short-term cell culture samples to normal brain tissue that had not been exposed to artificial cell culture conditions. However, the only alternative to this was cultured foetal astrocytes. Although this control has been exposed to artificial culture conditions, other sample parameters cause some concerns. This control is of foetal origin and as described when investigating the biopsy controls, the characteristics of foetal tissue, such as increased proliferation and reduced cell death are also associated with tumour growth and development. Moreover, only 2 sources of foetal astrocytes were available for this study.

As the foetal control was used in the investigation of paediatric astrocytoma short-term cell cultures with some caution, the experiment was not normalised directly to the controls. This allowed any comparisons made between tumour groups or grades, to be done so without the direct influence of controls, but also allowed comparisons to be made between the tumours and controls where appropriate. Moreover, the controls were effectively used as a baseline for some comparisons.

Another challenging aspect of this study was the analysis of 22000 gene expression results for every sample. Although tools in GeneSpring® can identify global gene expression profiles for each sample, isolate differentially expressed genes and cluster genes and samples accordingly; correlating these changes with tumour parameters and biologically significant patterns is still predominately determined by the investigator. This requires a vast amount of database mining to characterise gene ontologies, functions and pathways. Furthermore, many of the analysis approaches in GeneSpring® correlate expression patterns of hundreds of genes, a number that cannot be reliably investigated by database mining. Consequently in this study, differentially expressed genes were investigated by pathway or GOs to focus the analysis and where appropriate, key genes were further characterised by database mining. This allowed biologically relevant differential gene expression to be characterised in paediatric astrocytoma. Moreover, a molecular profile could not be linked to tumour or patient parameters, which is often the hypothesis in many studies.

It is understood that this not the only way to investigate this data set and in the hands of a different investigator, other biologically significant pathways or gene expression patterns may have been identified. Moreover, from this aspect, this data can be re-analysed at any time with an alternative hypothesis. This is also a positive feature of this technology and future expression patterns or genes, identified as significant in paediatric astrocytoma development, can be investigated in this sample cohort. It is also important to note that no software programme exists that can identify differential gene expression, define gene function, chromosome location and relevant information, accurately predict pathway involvements and alterations, highlight common biological processes and correlate all of this information into an overall result. Furthermore, CNAs or any other mechanism that may be relevant, also needs to be taken into consideration by any analysis software. Whilst this information is still correlated by the investigator it will be extremely difficult to understand the overall picture.

CNAs were extensively investigated in the paediatric astrocytoma of this study. All tumours showed single clone alterations, with specific tumours exhibiting multiple alterations of large chromosomal regions. Many of the single clone alterations were located in regions of known LCV in the normal population. Correlation of differential gene expression in these small regions of alteration was not always possible due to lack of reliable gene expression data. However, several candidate genes in regions of alteration in PAI biopsies were identified including, *BCL7B* at 7q11.23; *SH3GL2* at 9p21.2-p23; *BCL7A* at 12q24.33; *DRD1IP* at 10q26.3; *FOXG1B* at 14q12; and *TUBG2* and *CNTNAP1* at 17q21.31. In the short-term cell cultures comprised of all astrocytoma grades, candidate genes including *COL11A1* at 1p13.3; *FOXG1B* at 14q12; *IDI1* at 10p15.3; *EPHA2* at 1p35.1 and *ANXA8* at 10q11.22 were identified in 10 or more tumours. Although no reliable expression data was available for the genes located at 19p13.2; 16p11.2; 7p21; and 7q11.23, these regions have previously been associated with CNAs in other types of cancer. Moreover, single clone alterations at 14q12 and 7q11.23 were identified in both paediatric astrocytoma biopsy and short-term cell culture samples.

The detection of a large number of single clone alterations in specific tumours at regions of LCV, does suggest that these alterations may not be linked to tumour development. Moreover, if the alterations occur in a region of LCV at a higher frequency than that of

the normal population, this suggests that some LCV may predispose individuals to tumour development, such as alterations at 14q12.

In the biopsy and short-term cell culture studies investigating paediatric astrocytoma CNAs, the different grades involved in each experiment may have influenced the clone alteration frequencies. For example, loss at 14q12 was found in 82% of paediatric PAI biopsy samples, compared to gain in 28% and loss in 19% of paediatric astrocytoma short-term cell cultures of various grades. If biopsy samples had been available from additional grades, 14q12 alterations may have occurred at different in this experiment.

The resolution of aCGH has also been questioned with the theoretical sensitivity being estimated at 2.65Mb, the single-copy sensitivity being estimated at 2.65Mb and breakpoint precision being estimated at 4.55Mb (Coe et al., 2007). Essentially single copy alterations of approximately 50kb are detected in 15% of cases using the SpectralChip 2600, compared to alterations of approximately 500kb that are detected in 50% of cases. This is due to the BAC clone size and distribution along the genome, which is not uniform. Oligonucleotide based approaches are more sensitive detecting both 50kb and 500kb alterations in approximately 95% of cases (Coe et al., 2007). Recent experience in our laboratory has suggested that aCGH does not efficiently detect small regions of alteration in specific samples and that SNP arrays may be a more useful technique if small regions of alteration need to be clearly defined.

The dye swap approach completed in each aCGH experiment does provide confidence in single clone alteration detection in the paediatric astrocytoma samples of this study. However, it is essential that these alterations are validated using a different technique, such as SNP arrays or fluorescent *in situ* hybridisation (FISH), before any further investigations are completed.

Alterations of between 2 and 4 clones were identified in specific tumours and correlated with differential gene expression. From these results, it was hypothesised that differential gene expression at these loci may provide specific tumours with selective advantages. In total, 7 tumours showed whole chromosome and arm alterations in the paediatric astrocytoma short-term cell cultures. The majority of these tumours clustered in Group 3. Furthermore, differential gene expression that correlated with large CNAs

could be divided into two groups. Firstly, a large number of the differentially expressed genes correlated with the signature of 4682 probe sets that could distinguish the 3 tumour culture Groups. Secondly, those genes differentially expressed that did not correlate, had functions associated with tumour development and/or a function that could provide a specific tumour with a selective advantage, such as chemoresistance. This highlights tumour heterogeneity and a mechanism through which it may occur.

The most common whole chromosome or arm alteration seen in the tumours of this study was gain of part or all of 7q. This correlated with differential gene expression previously linked to tumour development, including astrocytoma. Moreover, gain of part or all of chromosome 7q has been previously associated with reduced patient survival.

The use of the reference control in the aCGH experiment has raised one question. The sex mismatch approach was recommended by the manufacturer for aCGH, in which the opposite sex DNA to the sex of the tumour patient is used as the reference control. However, it was felt that this may have hindered the investigation of sex chromosome alterations in the paediatric astrocytoma of this study and that it may have been more appropriate to adopt the sex paired approach, in which the same sex DNA to the sex of the tumour patient is used as the reference control. By using the sex mismatch approach the X chromosome was always shown as gained or lost. Whilst this was helpful for experimental analysis, it did not allow analysis of single clone or small regions of alteration that may have been present on the X chromosome, with the analysis approaches used here.

The large amount of unreliable gene expression results generated by the Affymetrix GeneChip® U133A was also very frustrating. Ultimately this reduced the number of correlations that could be made with CNAs. Technology has improved since the start of this project and the Affymetrix GeneChip® Plus 2 array is now available investigating approximately 50,000 gene sequences, in the future correlations between differential gene expression and CNAs may be more efficient. To ultimately determine whether gene expression is influenced by CNAs, all genes must be investigated along with every base pair in the genome, a tall order.

Affymetrix GeneChip® technology is designed to investigate global gene expression patterns and whilst reliable, this technology relies on experimental design. The investigation of brain tumours using this technology would only reach its full potential if multiple and repeated tumour sampling could be completed. This approach may be used to monitor tumour molecular changes associated with malignant progression or growth and tumour responses to treatment that may aid treatment regimes. Overall, multiple sampling of tumours would also improve characterisation of tumour regions, molecular profiles linked to astrocytomas in specific age groups and rare astrocytomas associated with unusual characteristics such as prolonged or reduced prognosis.

The survival data for patients in this study is some what compromised. Tumours involved in this study have been collected over a period of approximately 25 years. This has lead to significant variations in tissue collection procedures, patient diagnosis, patient treatment regimes and overall prognosis. Consequently, the survival data was not directly used in this study for statistical. Another tumour parameter significantly affected by the tumours being collected over a number of years is the grade at diagnosis. The review of tumour initial diagnosis according to the 2007 WHO grading scheme, re-graded 39% of the cases investigated. This did not affect the paediatric astrocytoma biopsy experiment, as all these tumours were PAI. However, in the paediatric short-term culture experiment, all reviewed DAII were re-graded as PAI and additional specific changes were also seen. Moreover, the new diagnoses according to the 2007 WHO grading scheme does correlate better with the tumour clusters according to molecular profile. Furthermore, a study in 2000, established that tumour diagnosis concordance between various neuropathologists was as low as 40% (Prayson et al., 2000). This raises the question of human error in tumour diagnosis and whether molecular profiling would be more efficient. However, there are undoubtedly difficulties in this approach on a global scale including, tumour sampling, tissue processing, experiment standardisations, characterisation of molecular profile features associated with various tumour and patient parameters and essentially cost.

Overall, these data remain descriptive with the aims of identifying novel candidate target genes and characterizing cellular pathways that are involved in paediatric astrocytoma development and growth. Future functional studies are needed to evaluate

these findings in paediatric astrocytoma but as discussed, evidence supports a role for these candidate genes and pathways in tumourigenesis.

The overall conclusions of this study are that paediatric astrocytoma biopsies and short-term cell cultures can be used in the investigation of this tumour, but they cannot be directly compared and specific controls for each experiment must be used. Young adult normal total brain is a reliable control for the biopsy experiment although if a paediatric normal total brain sample was available this may be more appropriate. Foetal astrocytes were an acceptable control for the short-term cell culture experiment, but comparisons between other culture controls such as adult astrocytes would have strengthened this control choice.

It was not possible to identify expression profiles or molecular signatures of any tumour or patient parameters, mainly because of the sample numbers. When tumour location or patient age was investigated, no molecular profile could be identified with confidence that distinguished sub-groups within these parameters. Correlations between grade and molecular profile were improved after diagnoses review.

Molecular pathways involved in paediatric astrocytoma development could be clearly identified, with pathway alterations in Group 3 characterising a more malignant tumour. CNAs were identified in paediatric astrocytoma that did correlate with differential expression and could be a mechanism disrupting gene expression. However, gene promoter methylation was not found to be involved in differential gene expression.

CHAPTER 7

FUTURE WORK

To expand this study investigating paediatric astrocytoma validation of CNAs and differential gene expression in regions of interest would first need to be completed. Moreover, this approach may also include genes in regions of CNA that did not have reliable expression data. Gene expression validations could be completed by Q-PCR analysis and CNAs through SNP arrays, FISH or m-FISH (multiplex fluorescent *in situ* hybridisation) analysis. SNP arrays have a higher resolution compared to aCGH and use an oligonucleotide based approach (Coe et al., 2007). This technique may also define the regions of interest further.

FISH is a physical mapping approach that uses fluorescent tags to detect the hybridization of probes with metaphase chromosomes. In this case, specific probes complementary to regions of CNAs of interest would be used. Furthermore, m-FISH can be used to identify simple and complex translocations, interstitial deletions and insertions, chromosomal aneusomies and double minutes. The technique uses combination labelling with between 1-5 fluorochromes for each chromosome, providing every chromosome with a specific spectral signature. Both latter approaches allow single cell analysis, compared to total sample analysis adopted by aCGH or SNP arrays and may highlight tumour heterogeneity as well as common alterations.

Aspects of this study may need to be refined for future work in order to reduce variability between tumour samples and also generate a wider picture of tumour molecular profiles. Multiple biopsies of a single tumour have been used to investigate variations in tumour molecular profile at different tumour sites, including tumour core and periphery (Van et al., 2006). The same approach has also been used to investigate variations in gene mutations within tumour regions, including the frequency and variability of *TP53* mutations in a single tumour (Watanabe et al., 1997a). Unfortunately, this approach requires imaging and surgical approaches that may be difficult to implicate, given that material currently received is surplus to diagnosis.

Microdissection is another approach previously used to investigate histophorically different tumour regions, as well as specific regions such as proliferating stromal blood vessels, within a given sample. This technique allows the selection of tumour cells by

region or type prior to investigation with further techniques such as aCGH or FISH. This allows the exact location/frequency of specific alterations within individual tumour regions or cells to be characterised (Romeike et al., 2001; Kulla et al., 2003; Wemmert et al., 2006).

A large number of single clone alterations in the tumours of this study were found in regions of LCVs that occur in the normal population. It was not possible to determine whether the CNAs found within the tumours, where also somatic alterations. Paired patient blood DNA is available for the majority or tumours investigated and it would be valuable to identify those clones that are altered in the tumour only compared to germ line controls, as well as alterations that occur in both tumour and germ line DNA at a higher frequency than the normal population. This may identify DNA variants in the normal population that could predispose individuals to tumour development, such as the CNA identified at 14q12 (Braude et al., 2006). A similar approach has been carried out in GBMIV (Balakrishnan et al., 2007).

3D cell culture models are being investigated as a more appropriate approach to studying tumour cell behaviour *in vitro*. It has been suggested that cells cultured in a 3D environment are more representative of tumour cells *in vivo*. The paediatric astrocytoma short-term cell cultures studied here are a valuable resource and the use of 3D culture environments could be an essential step in the study of these tumours, given the limited availability of biopsy material. This approach has already been described using malignant breast epithelial cells (Lee et al., 2007a).

A limiting factor in this study is the number of tumours available for investigation. This is a reflection of the rarity of this tumour, preventing the analysis of parameters such as patient age or tumour location and specific molecular signatures. Furthermore, validation of the tumour clusters seen here could not be completed on an independent tumour group as the samples and funding were not available. Guidelines for array experiments with respect to platform and controls would allow more comparisons between tumours of different studies, although this would be difficult to implement. Moreover, it would be particularly valuable if this study could be on-going and include any newly diagnosed paediatric astrocytoma. Overtime, this would increase experiment numbers allowing a more detailed analysis and validation of initial conclusions.

Tumour grade was a difficult parameter to investigate in this study. The initial grading criteria used to diagnose these tumours, was outlined by the WHO prior to 2003. However, due to the new WHO grading scheme released in 2007 the initial diagnosis of the majority of tumours was reviewed in August 2007. A large proportion of the initial diagnoses were altered to correlate with the new grading scheme. This raises several questions regarding tumour grading according to histological criteria and how to approach the continuous review of the criteria used to define each astrocytoma grade. This also highlights the need for a tumour molecular profile based diagnostic approach, in conjunction with histopathological grading and current immunohistochemical staining. From the experience gained during this report, all tumour diagnoses should be reviewed prior to the start of a study and when new guidelines are issued. Changes are inevitable that may influence analysis approaches and conclusions.

Many of the tumours investigated in this study showed only single clone alterations, suggesting that additional mechanisms are involved in the development of specific paediatric astrocytoma. MSP was used to investigate specific genes commonly methylated in other tumour types. However, promoter region methylation was not identified in any of the astrocytomas investigated here. Furthermore, the methylation status of *MGMT* has been linked to treatment response to temozolomide, highlighting this significant mechanism in tumour growth and development. To fully investigate this mechanism in paediatric astrocytoma, a combined approach of pharmacologic inhibition of DNA methylation and histone deacetylation involving 5-aza-2'-deoxycytidine and trichostatin A treatment, coupled with expression microarrays, could identify global methylation patterns distinct to normal controls. This is already being investigated in adult GBMIV (Kim et al., 2006).

To continue the work compiled in this study, genes with strong evidence that suggests a role in tumour development, could be investigated further in expression manipulation studies. This may define cellular response and clarify the role of specific genes in paediatric astrocytoma with the aim of generating therapeutic targets. Candidates for investigation would include genes differentially expression in regions of CNAs, genes showing large changes in expression compared to controls and differentially expressed genes involved in a large number of pathways. Similar studies have already been completed including the use of retroviral constructs and adenoviral expression

constructs to investigate the influence of tyrosine kinase pyk2 on GBMIV cell migration (Lipinski et al., 2005). Furthermore, siRNA directed against the unique exon 1/exon 8 junction sequence of the most frequently occurring EGFR mutation in astrocytoma efficiently suppresses the expression of the mutant receptor in rodent fibroblasts and two human glioblastoma cell lines. siRNA-mediated depletion of the mutant EGFR led to reduced levels of phosphorylated Akt in glioma cells, that was associated with increased apoptosis and partial cell cycle arrest in the G2M phase (Fan and Weiss, 2005).

Overall, the multiple CNAs and differential gene expression identified in this study suggest that many relevant pathways are involved in tumour predisposition and development, although establishing the exact role of each CNA or differentially expressed gene identified was beyond the scope of this study. Moreover, the characterisation of a larger number of differentially expressed genes and CNAs in the tumour cultures of Group 3, which consists predominately of high-grade cultures, may suggest that multiple sequential alterations have occurred in these tumours compared to the tumours of Group 1 and 2. This is also seen in the progression of adult astrocytoma from DAII to GBMIV with the accumulation of specific alterations and differential expression (Figure 1.8).

A nation wide study of somatic DNA from patients diagnosed with a paediatric astrocytoma could identify and confirm the role of specific LCVs in astrocytoma predisposition. This approach would also validate the role of CNAs at 14q12 and the resulting differential expression of *FOXG1B*, suggested in this study, in the predisposition of paediatric astrocytoma. No doubt distinct alterations predispose different individuals to tumour development. Multiple biopsy samples taken from patients at diagnosis and at multiple time points thereafter would allow CNA and differential expression to be investigated as the tumour develops. This would establish which CNA and gene expression alterations are involved in early tumour development compared to the latter stages. Additional studies including those previously mentioned investigating methylation status and epigenetic disruption would define the involvement of these mechanisms in tumour development at specific stages. This approach is not novel and was initially investigated by Cavenee et al. (1991). However, this approach

would need to be carried out routinely and encompass a large number of patients to establish significant alterations at specific stages of tumour development.

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APPENDIX

APPENDIX I

Reagents and buffers

Media

The cell culture media was made up in batches of 9, 500ml volumes of Hams F10 nutrient mix with 10% FCS. Initially, 9 Hams F10 bottles were labelled A-I and allowed to reach room temperature. Universals were also labelled A-I and FCS. 5ml of FCS was pipetted into the correct universal. In turn 55ml of FCS was pipetted into a bottle of Hams F10, the bottle mixed and 10ml transferred to the correct universal. The media was then stored at 4°C until needed. The universals were incubated at 37°C for 3 days to ensure no contamination had occurred.

Antibiotics

The final concentration of antibiotics required per ml of media was as follows; penicillin/Streptomycin 100U/100µg per ml, kanamycin 50µg/ml, amphotericin 2.5µg/ml. To a 1.5ml tube, 0.5ml of the penicillin/Streptomycin original solution (10,000U/10mg per ml), 25µl of the amphotericin original solution (5mg/ml) and 0.25ml of kanamycin original solution (10mg/ml) were added. The antibiotic mix was stored in at -20°C. When needed the mix is added to 50ml of media to reach the correct concentration

Biopsy tissue collection media

Media without FCS containing antibiotic at the previous concentration described was used to collect the biopsy samples from theatre. Therefore, 1 tube of antibiotic mix (previously described) was added per 50ml of FCS free media.

Agilent –dye preparation

To prepare the gel-dye mix, 0.4ml of gel mix was placed in the provided filter and centrifuged at 4000rpm for 10 minutes. From the elution, 130µl was add to 2µl of concentrated RNA dye and vortexed thoroughly. The gel-dye mix was used within 1 week and the gel mix was used within 1 month as recommended by the manufacturer.

DEPC-treated water

1ml of diethylpyrocarbonate (DEPC) was added to 1L of water. This was then shaken vigorously, autoclaved and stored at room temperature.

NaCL

1M = 58.44g/L
For 3M NaCL and 5M NaCL, 17.53g and 29.22g were dissolved in 100ml DEPC-treated water respectively. The solutions were then autoclaved and stored at room temperature.

0.5M Na_2EDTA Stock

186.12g of disodium ethyenediaminetetraacetate was dissolved in 800ml DEPC-treated water. The pH was adjusted to pH8.0 with NaOH and the total volume made up to 1L. This was autoclaved and stored at room temperature.

50x TAE buffer

242g of Tris was added to 500ml of DEPC-treated water. 100ml of 0.5M Na_2EDTA and 57.1ml of glacial acetic acid was added and the total volume adjusted to 1L. This was autoclaved and stored at room temperature.

Ethidium bromide stock

The stock solution concentration was 10mg/ml. This was made by adding 1g of ethidium bromide to 100ml of DEPC-treated water. This was stirred until the dye dissolved and stored in the dark at room temperature.

DNA loading dye

Prepared in DEPC-treated water; 50% glycerol, 0.25% xilene cyanol, 0.25% bromophenol blue and 1% SDS. This was stored at -20°C.

RNA loading dye

Prepared in DEPC-treated water; 50% glycerol, 0.4% bromophenol blue and 1mM Na_2EDTA . This was then stored at -20°C.

Qiagen kit buffers

The majority of Qiagen buffers were already prepared. However, buffer RPE was supplied as a concentrate and the correct volume of absolute ethanol was added before use. This amount depended on the kit being used. β-mercaptoethanol was added to buffer RLT before use at 10µl per 1ml.

Buffer C1 (cell lysis buffer)
1.28M sucrose; 40mM Tris-Cl; 20mM $MgCl_2$; 4% Triton X-100.
Initially, 438.14g sucrose, 406g $MgCl_2$ and 4.84g Tris base was dissolved in 680ml DEPC-treated water and 42g of Triton X-100 (100%) added. The pH was adjust to pH pH7.5 and the volume made up to 1L with DEPC-treated water.

Buffer G2 (digestion buffer)
800mM guanidine-HCl; 30mM Tris-Cl; 30mM EDTA; 5% Tween-20; 0.5% Triton X-100.
Initially, 76.42g guanidine-HCl, 11.17g Na_2EDTA and 3.63g Tris base was dissolved in 600ml DEPC-treated water and 250ml of 20% Tween-20 solution and 50ml of 10% Triton X-100 solution was added. The pH was adjusted to pH 8 and the volume made up to 1L with DEPC-treated water.

Buffer QBT (equilibration buffer)
750mM NaCl; 50mM MOPS; 15% isopropanol; 0.15% Triton X-100.
Initially, 43.83g NaCl and 10.46g MOPS was dissolved in 800ml DEPC-treated water. The pH was adjusted to pH 7 and 150ml of pure isoprpanol and 15ml of 10% Triton X-100 added. The volume was then made up volume to 1L with DEPC-treated water.

Buffer QC (wash buffer)
1M NaCl; 50mM MOPS; 15% isopropanol.
Initially, 58.44g NaCl and 10.46g MOPS was dissolved in 800ml DEPC-treated water. The pH was adjusted to pH 7 and 50ml of pure isopropanol added. The volume was made up to 1L with DEPC-treated water.

Buffer QF (elution buffer)
1.25M NaCl; 50mM Tris-Cl; 15% isopropanol.
Initially, 73.05g NaCl and 6.06g Tris Base were dissolved in 800ml DEPC-treated water. The pH adjusted to pH 8.5 and 150ml pure isopropanol added. The volumewas made up to 1L with DEPC-treated water.

Sample clean-up module buffers

The majority of Sample Clean-up Module buffers were already prepared. However, cDNA binding buffer was supplied as a concentrate and 24ml of absolute ethanol was added before use.

10M NaOH

Initially, 4g of NaOH was dissolved in 10ml DEPC-treated water. For 1M, 100µl of the 10M solution was added to 900µl of DEPC-treated water. For 3M, 150µl of the 10M solution was added to 350µl of DEPC-treated water.

10mM Hydroquinone

Initially, 0.220g was dissolved in 20ml of DEPC-treated water.

3M sodium bisulfite

Initially, 3.183g of sodium bisulfite was dissolved in 9.5ml of DEPC-treated water and 360µl NaOH added before making the final volume up to 10ml.

10X MSP buffer

This was made up according to the Table below.

	Volume (ml)	10X Stock
1M Ammonium Sulfate	8.3	16.6mM
1M Tris/HCL	33.5	67mM
1M $MgCL_2$	3.35	6.7mM
β-Mercaptoethanol (14.3M)	0.35	10mM
Total Volume	50	

12.5mM NTP

This was made up according to the Table below.

	Volume (µl)
100mM dATP	12.5
100mM dCTP	12.5
100mM TTP	12.5
100mM GTP	12.5
DEPC-treated water	50
Total Volume	100

12X MES stock buffer

64.61g of MES hydrate and 193.3g of MES sodium salt were added to 800ml of DEPC-treated water. The pH was checked to ensure that it was between pH6.5-6.7 and the volume adjusted to 1L. This was then stored at 4°C.

2X hybridisation buffer

8.3ml of 12X MES stock buffer was added to 19.9ml of DEPC-treated water. 17.7ml of 5M NaCL was then added followed by 4ml 0.5M EDTA and 0.1ml of 10% Tween-20 to give a final volume of 50ml. This was then stored out of direct sunlight at 4°C.

Wash buffer A: non-stringent wash buffer

For a 1L final volume, 300ml of 20X SSPE was added to 699ml of DEPC-treated water and 1ml of 10% Tween-20. This was then stored out of direct sunlight at 4°C.

Wash buffer B: stringent wash buffer

For a 1L final volume, 41.7ml of 12X MES stock buffer was added to 910.5ml of DEPC-treated water along with 5.2ml 5M NaCL, and 1ml of Tween-20. This was then stored out of direct sunlight at 4°C.

2X stain buffer

For a final volume of 250ml, 41.7ml of 12X MES stock buffer was added to 113.3ml of DEPC-treated water, along with 92.5ml of %M NaCL and 2.5ml of 10% Tween-20. This was then stored out of direct sunlight at 4°C.

10mg/ml Goat IgG stock

50mg of goat IgG was resuspended in 5ml of 150mM NaCL and stored at 4°C.

Antibody solution

The antibody solution was made up as shown in the table below and used the same day.

Components	Volume	Final Concentration
2X Stain Buffer	300µl	1X
50mg/ml BSA	24µl	2mg/ml
10mg/ml Goat IgG Stock	6µl	0.1mg/ml
0.5mg/ml Biotinylated Antibody	3.6µl	3µg/ml
DEPC-Treated Water	266.4µl	-
Total Volume	600µl	

SAPE stain solution

Streptavidin Phycoerythrin (SAPE) solution was made up as shown in the table below, stored at 4°C foil wrapped and used the same day.

Components	Volume	Final Concentration
2X Stain Buffer	600µl	1X
50mg/ml BSA	48µl	2mg/ml
1mg/ml SAPE	12µl	10µg/ml
DEPC-Treated Water	540µl	-
Total Volume	1200µl	

Assays-On-Demand – Applied Biosystems

CCNA1: Hs 00171105_m1
SPINT2: Hs 00173936_m1
MAPK1: Hs 00177066_m1
MATK: Hs 00176738_m1
TIMP1: Hs 00171558_m1
MCL1: Hs 00172036_m1

20X SSC (saline-sodium-citrate buffer)

Initially,175.3g of sodium chloride and 88.23g of tri-sodium citrate was dissolved in 1L of DEPC-treated water.

0.2X SSC

50ml of 20X SSC was added to 450ml DEPC-treated water.

2X SSC, 0.5% SDS

Initially, 2.5g of SDS (sodium dodecyl sulphate) was dissolved in 12.5ml DEPC-treated water. This was added to 437.5ml DEPC-treated water and 50ml 20X SSC.

2X SSC, 50% deionised formamide

250ml of deionised formamide was added to 200ml DEPC-treated water and 50ml 20X SSC.

2X SSC, 0.1% igepal

Initially, 1ml of igepal was dissolved in 4ml DEPC-treated water. 2.5ml of this solution was added to 447.5ml DEPC-treated water and 50ml 20X SSC.

Supplier information

Product	Supplier
Plastics	Scientific Laboratory Supplies, Wilford Industrial Estate, Nottingham, NG11 7EP. UK www.scientific-labs.com Triple Red Ltd, Unit D4 Drakes Park, Long HP18 9BA. UK www.triplered.com
Chemicals	Sigma Aldrich, Fancy Rd, Poole, Dorset, BH12 4QH. UK www.sigmaaldrich.com
Total RNA Samples	ams Biotechnology, 63B Milton Park, Abingdon, Oxon, OX14 4RX. UK www.amsbio.com
Total RNA Samples	United States Biological, P.O. Box 261, Swampscott, Massachusetts 01907. www.usbio.net
Total RNA Samples	BD Biosciences Clontech, 1020 East Meadow Circle, Palo Alto, CA 94303-4230. www.bdbiosciences.com
Normal Human Astrocytes	Cambrex Bio Science, Rockland, ME USA. www.cambrex.com
RNase AWAY	Molecular BioProducts Inc. San Diedo, CA www.mbpinc.com
RNeasy Column Kits	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com
TRIzol	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
QIAamp® DNA Mini Kit	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com
Proteinase K	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com
Protease	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com

Qiagen Genomic-tips 100/G	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com
Hams F10 Nutrient Mix	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Foetal Bovine Calf Serum	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Trypsin EDTA	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Ispton II	Beckman Coulter Counter Ltd, Kingsmead Business Park, High Wycombe, HP11 1JU. UK www.beckmancoulter.com
Coulter Counter	Beckman Coulter Counter Ltd, Kingsmead Business Park, High Wycombe, HP11 1JU. UK www.beckmancoulter.com
Glass Pasteurs	Fisher Scientific UK, Bishops Meadow Rd, Loughborough, LE11 5RG. UK www.fisher.co.uk
Primers	Amersham Bioscience, Amersham Place, Little Chalfont, Bucks, HP7 9NA. UK www5.amershambiosciences.com
Taq Polymerase	Promega, Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, S09 1BG. UK www.promega.com
Ultrapure dNTP set	Amersham Bioscience, Amersham Place, Little Chalfont, Bucks, HP7 9NA. UK www5.amershambiosciences.com
Filter Pipette Tips (RNase/DNase free)	Fisher Scientific UK, Bishops Meadow Rd, Loughborough, LE11 5RG. UK www.fisher.co.uk
Thermal Cycler	Genetic Research Instrumentation (GRI) Ltd, Gene House, Queensborough Lane, Braintree, Essex, CM7 8TF. UK www.gri.co.uk
T7 Oligo(dT) Primer	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com

Superscript Choice System	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
GeneChip Sample Cleanup Module	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
GeneChip® Human Genome U133A	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
GeneRuler™100bp DNA Ladder	MBI Fermantas, (Helena Biosciences Europe), Colima Avenue, Sunderland Enterprise Park, Sunderland, SR5 3XB. UK www.fermantas.com.
Enzo Bioarray High Yield RNA Transcript Labeling Kit	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
RNA Centaury Marker	Ambion Ltd, Ermine Business Park, Spitfire Close, Huntingdon, Cambridgeshire. PE29 6XY. UK www.ambion.com
QuantiTect® Reverse Transcription Kit	Qiagen, Boundard Court, Gatwick Rd, Crawley, West Sussex, RH10 9A. UK www.qiagen.com
Assays-on-Demand™	Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA. 94404. www.appliedbiosystems.com
Herring Sperm DNA	Promega, Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, S09 1BG. UK www.promega.com
GeneChip Eukaryotic Hybridisation Control Kit	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
R-Phycoerythrin Streptavin	Molecular Probes, (Invitrogen Ltd), 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.probes.com
Phosphate Buffered Saline	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Mineral Oil	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
20X SSPE	Cambrex Bio Science, Rockland, ME USA. www.cambrex.com

Goat IgG, Reagent Grade	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
Anti-streptavidin Antibody (Goat) Biotinylated.	Vector Laboratories Ltd, 3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS. UK www.vectorlabs.com
Fluidics Station 450	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
GeneChip scanner 3000.	Affymetrix Inc, 3380 Central Expressway, Santa Clara, CA. www.affymetrix.com
GeneSpring version 6.1	Silicon Genetics, 2601 Spring Street, Redwood City, CA 94063 www.silicongenetics.com
Plasmocin™	Autogen Bioclear UK Ltd, Holly Ditch Farm, Mile Elm Calne, Wiltshire, SN11 0PY. UK www.autogenbioclear.com
MycoAlert™	Cambrex Bio Science, Rockland, ME USA. www.cambrex.com
Kanamycin	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
Amphotericin	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
Penicillin/Streptomycin	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
Polyclonal Rabbit anti-GFAP	DakoCytomation, Produktionsvej 42, DK-2600, Denmark www.dakocytomation.com
Polyclonal Goat anti-Rabbit	DakoCytomation, Produktionsvej 42, DK-2600, Denmark www.dakocytomation.com
Vectastin® ABC method	Vector Laboratories Ltd, 3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS. UK www.vectorlabs.com
3,3'-diaminobenzidine (DAB)	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
HistClear	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com

D.P.X	Sigma Aldrich, Fancy Rd, Poole, Dorset BH12 4QH. UK www.sigmaaldrich.com
Centrifuge (Biofuge Pico)	DJB Labcare, 20 Howard Way, Interchange Business Park, Newport Pagnell, Bucks, MK16 9QS UK www.djblabcare.co.uk
Centrifuge (Wifug 500E)	DJB Labcare, 20 Howard Way, Interchange Business Park, Newport Pagnell, Bucks, MK16 9QS. UK www.djblabcare.co.uk
Class II Laminar Flow Cabinet	Valeant Plaza, 3300 Hyland Drive, Costa Mesa, California 92626. USA www.valeant.com
Fume Cabinet (Astecair 500E)	Bioquell UK Ltd, 34 Walworth Road, Andover, Hants, SP10 5AA. UK www.bioquell.com
Luminometer (Mediators PhL)	Aureon Biosystems GmbH, Simmeringer Hauptstr. 24 A-1110 Vienna / Austria www.aureonbio.com
0.5M EDTA	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Superfrost slides	Fisher Scientific UK, Bishops Meadow Rd, Loughborough, LE11 5RG. UK www.fisher.co.uk
Wizard® DNA Clean-Up System	Promega, Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, S09 1BG. UK www.promega.com
PureLink™ Quick Gel Extraction Kit	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Sequnencing and Reaction Buffer	Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA. 94404. www.appliedbiosystems.com
Montage™ PCR Centrigugal Filter Devices	Millipore, 290 Concord Road Billerica, MA. 01821 USA. www.millipore.com
Spectrophotometer (GeneQuant)	Pharmacia Biotech, Amersham Bioscience, Amersham Place, Little Chalfont, Bucks, HP7 9NA. UK www5.amershambiosciences.com

SpectralChip™ 2600 arrays	GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA. UK. www.gehealthcare.com
Cy3-dCTP and Cy5-dCTP	GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA. UK. www.gehealthcare.com
Spectral Hybridisation Buffers	GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA. UK. www.gehealthcare.com
Bioprime Labelling Kit	Invitrogen Ltd, 3 Foundation Drive, Inchianan, Renfrewshire, PA9 9FR. UK www.invitrogen.com
Dessiccator	Nalge Company, A Subsidiary of Sybron Corporation, P.O.Box 20365, Rochester, NY, 14602-0365. USA.
Hybridisation Chamber	Corning Life Sciences, Koolhovenlaan, 12 NL-1119, NE Schiphcl-RIJK, The Netherlands.
22X60mm Coverslips	Electron Microscopy Sciences, P.O.Box 550, 1560 Industry Road, Hatfield, PA, 19440. USA www.emsdiasum.com
Axon 4000A Scanner	Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, CA, 94089-1136, U.S.A. www.moleculardevises.com
Gene PixPro version 6.1	Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, CA, 94089-1136, U.S.A. www.moleculardevises.com
Formatter Software	Digital Scientific UK, Sheraton House, Castle Park, Cambridge, CB3 0AX. UK www.digitalscientific.co.uk
Human Genomic DNA	Promega, Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, S09 1BG. UK www.promega.com

APPENDIX II

Patient treatment regimes and survival for the paediatric astrocytoma of this study.

IN[1]	Treatment[2]	Survival[3]
1350	Macroscopic removal	Unknown
1520	Partial excision, radiotherapy	DIS 112 (A)
1524	Macroscopic removal, VP shunt	13 (A)
1591	Craniotomy, partial excision	60 (D)
1740	Macroscopic removal	160 (A)
2017	Macroscopic removal	124 (A)
2110	Macroscopic removal	DIS 125 (A)
2122	Partial excision, radiotherapy, VP shunt	DIS 78 (A)
2356	Macroscopic removal	142 (A)
2368	Macroscopic removal	DIS 87 (A)
2524	Macroscopic removal	DIS 39 (A)
2604	Unknown	Unknown
2631	Surgery	21 (A)
2674	Macroscopic removal	112 (A)
2688	Craniotomy, macroscopic removal	55 (A)
2788	Macroscopic removal	45 (A)
2825	Surgery x2, cranial radiotherapy	104 (A)
2921	Macroscopic removal, LGG (wait & see)	48 (A)
2940	Macroscopic removal, LGG (wait & see)	74 (A)
2969	VP shunt, Macroscopic removal, LGG (wait & see)	57 (A)
3013	Craniotomy, subtotal removal, LGG (wait & see)	58 (A)
3017	Craniotomy, sub total removal, LGG (wait & see)	59(A)
3115	VP shunt, debulking, LGG (wait & see)	33 (A)
3126	Subtotal Removal (x3), Radiotherapy, craniotomy (x2)	31 (A)
3156	Surgery	1 (A)
380	Macroscopic removal, radiotherapy	92 (D)
1145	Macroscopic removal	60 (A)
1751	Craniotomy, macroscopic removal, VP shunt, radiotherapy	63 (A)
1869	Craniotomy, partial excision, radiotherapy	139 (A)
1930	Temporal lobectomy	53 (A)
2012	Partial excision, radiotherapy	145 (D)
2044	Craniotomy, macroscopic removal	DIS 18 (A)
2102	Partial excision, radiotherapy	67 (A)
3032	Partial excision, radiotherapy, VP shunt, craniotomy for debulking	12 (D)
2003*	Macroscopic removal, craniotomy	117 (A)
2591*	Radiotherapy	117 (A)
178"	Unknown	3 (D)
179"	Unknown	3 (D)
699	Stereotactic biopsy	Unknown
1163	Craniotomy, macroscopic removal	DIS 153 (A)
1262	Removal (extent unknown), radiotherapy	12 (A)
1419**	Partial excision, radiotherapy	12 (D)
1495	Removal (extent unknown)	80 (A)
1523	Unknown	33 (D)
1566	Partial removal	7 (A)
2087	Craniotomy, partial excision, cranial radiotherapy	(D)
2675	Craniotomy, partial excision, chemo (combined), cranial radiotherapy	16 (D)
3046	Partial excision, radiotherapy	14 (D)
2435	Unknown	Unknown
2731	Unknown	Unknown
2810	Unknown	Unknown
1760	Unknown	12 (D)
1265	Unknown	15 (D)
1528	Unknown	7 (D)
2234	Unknown	Unknown

IN[1]: Institute of Neurology assigned number; Treatment[2]: LGG = low-grade glioma protocol; BB = baby brain protocol (Appendix II); Survival[3]; survival in months from the data of diagnosis, A = alive; D = deceased. DIS = Patient has been discharge; * and " = tumours from the same patient; ** = patient diagnosed with Turners Syndrome (partial or complete loss of chromosome X).

Clinical Protocols for the treatment of patients in this study.

Baby brain protocol (UKCCSG CNS 9204)

This protocol was used to treat patients below 3 years of age with malignant astrocytoma. After surgery, patients received vincristine, carboplatin, methotrexate, cyclophosphamide and cisplatin over a 43 day cycle with a maximum of 7 cycles, a total treatment time of 379 days.

Dose schedule:	Vincristine	$1.5mg/m^2$ x 1
	Carboplatin	$550mg/m^2$ x 1
	Methotrexate	$8g/m^2$
	Cyclophosphamide	$1500mg/m^2$ x 1
	Cisplatin	$40mg/m^2$ x 1

Low-grade glioma study

This protocol was used to treat patients with low-grade astrocytoma. After surgery patients underwent one of two arms of treatment in this study. The first arm was the "wait and see" arm, in which patients only had further treatment if they had residual bulky disease, initial symptoms were not relieved by surgery or if recurrence or tumour progression occurred. The second arm of the study was the "treatment" arm, in which patients received carboplatin and vincristine. Initial treatment lasted 10 weeks with administration of vincristine on the first day of each week and carboplatin in weeks 1, 4, 7 and 10. After a 2 week recovery period, vincristine and carboplatin were administered together in weeks 13, 17, 21, 25, 29, 33, 37, 41, 45 and 49. Doses were reduced by 33% for patients 6 months of age or less.

Dose schedule:	Carboplatin	$550mg/m^2$ x 1
	Vincristine	$1.5mg/m^2$ x 1

Additional Protocols

Metaphase spread preparation

From a stock solution of 10μg/ml, 42μl of colcemid was added to a 75cm^3 flask of 80% confluent cells and incubated at 37°C for 2-3 hours. The flask content was pipetted into a sterile universal and centrifuge at 1500rpm for 5 minutes to collect the pellet. Initially, the flask was trypsinised as previously described, the 10ml of media containing the cells added to the pellet and the universal was centrifuged at 1500rpm for 5 minutes. The supernatant was removed, the pellet transferred to a falcon tube and 10ml of 0.3% sodium chloride (NaCL) added. This solution was incubated at room temperature for 30 minutes.

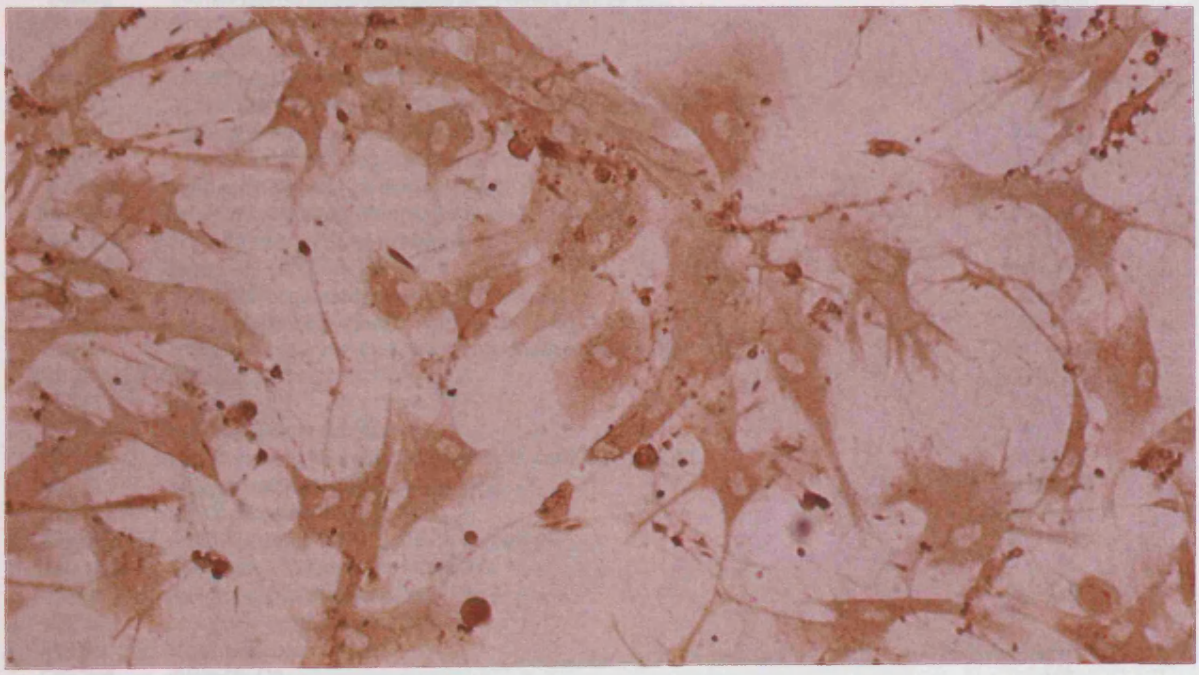
Once incubated, the flacon tube was centrifuged at 1500rpm for 5 minutes, the supernatant removed, 10ml of fix (75% methanol, 25% acetic acid) added and the solution incubated for 5 minutes. The mixture was centrifuged at 1500rpm for 5 minutes and the step repeated twice with an incubation of 30 minutes. The final pellet was resuspended in 1ml of fix. Superfrost slides (Fisher Scientific, UK) were placed in absolute alcohol for a minimum of 2 minutes, removed, allowed to air dry and labelled. One drop of the fix/cell solution was dropped onto the centre of the slide, the slide flooded with fix after 10 seconds and allowed to dry.

Giemsa Stain (Sigma Aldrich) was used to stain the slide involving a 10 minute incubated at room temperature. The slide was rinsed using distilled water and allowed to air dry. Metaphase spreads were evaluated using light microscopy. Any remaining fix/cell solution was stored at -20°.

Glial fibrillary acidic protein staining

Cells were grown on coverslips in 24 well plates until confluent. The cells were then fixed in para for 30 minutes, followed by two 5 minute washes in 1X phosphate buffered saline (PBS) in preparation for staining. The fixed coverslips were washed with 1X PBS and 0.1% Triton for 15 minutes, followed by blocking in 1X PBS, 3% normal goat serum and 5% powdered milk for 1 hour. The coverslips were washed twice with 1X PBS and 0.1% triton for 5 minutes, and with 1X PBS for a further 5 minutes. The primary anti-body (polyclonal rabbit anti-GFAP, DakoCytomation) was applied to the coverslips over night at a dilution of 1:100 in 1X PBS. The coverslips

were washed twice as previously described. The secondary-biotinylated antibody (polyclonal goat anti-rabbit, DakoCytomation) diluted 1:100 in 1X PBS, was applied to the coverslips and incubated for 2 hours. The coverslips were incubated in standard ABC solution for 1 hour (Vectastin® ABC method, Vector Laboratories) followed by 3 washes with 1X PBS each for 5 minutes. To stain the coverslips, 3,3'-diaminobenzidine (DAB) solution (SIGMAFAST™ DAB tablets, Sigma Aldrich) was applied and removed as soon as brown colouration was observed. The coverslips were washed 3 times with 1X PBS for 5 minutes, washed consecutively in 70% alcohol, 90% alcohol and twice in absolute alcohol. Two final washes were carried out in HistClear (Sigma Aldrich) for 2 minutes, before the coverslips were removed from the 24 well plates and mounted on clean slides using D.P.X (Sigma Aldrich). The extent of cell staining was evaluated using light microscopy.



This Figure illustrates the staining of GFAP in GP-9, a sample of foetal astrocytes.

APPENDIX III

The genes found in Figure 3.7 of Chapter 3 in dendrogram order.

The green lines are in the same place as those shown on the dendrogram of Figure 3.7.

Gene Name	Description	Chromosome Location
ABL1	v-abl Abelson murine leukaemia viral oncogene homolog 1	9q34.1
PPP1R15A	protein phosphatase 1, regulatory subunit 15A	19q13.2
TGFB1	transforming growth factor, beta 1	19q13.2
PPP6C	protein phosphatase 6, catalytic subunit	9q34.11
RHOQ	ras-like protein TC10	2p21
CDC23	CDC23 (cell division cycle 23, yeast, homolog)	5q31
KPNA2	karyopherin alpha 2	17q23.1-q23.3
CHC1	chromosome condensation 1	1p36.1
FLJ42280	Deleted in split-hand/split-foot 1 region	7q21.3-q22.1
KPNA2	karyopherin alpha 2	17q23.1-q23.3
KIAA0010	ubiquitin-protein isopeptide ligase (E3)	7q36.3
TP53	tumor protein p53 (Li-Fraumeni syndrome)	17p13.1
CDCA4	cell division cycle associated 4	14q32.33
CUL5	cullin 5	11q22-q23
PLEKHC1	mitogen inducible 2	14q22.1
IRF1	interferon regulatory factor 1	5q31.1
NBL1	neuroblastoma, suppression of tumorigenicity 1	1p36.3-p36.2
NEK9	NIMA (never in mitosis gene a)- related kinase 9	14q24.2
FGF2	fibroblast growth factor 2 (basic)	4q26-q27
AHR	aryl hydrocarbon receptor	7p15
CDC37L1	Hsp90-associating relative of Cdc37	
LTBP2	latent transforming growth factor beta binding protein 2	14q24
MN1	meningioma (disrupted in balanced translocation) 1	22q12.1
UBE2V1	ubiquitin-conjugating enzyme E2 variant 1	20q13.2
MAPK6	mitogen-activated protein kinase 6	15q21
NHP2L1	NHP2 non-histone chromosome protein 2-like 1	22q13.2-q13.31
SH3BP4	SH3-domain binding protein 4	2q37.1-q37.2
EXT2	exostoses (multiple) 2	11p12-p11
UBE2V1	ubiquitin-conjugating enzyme E2 variant 1	20q13.2
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6p21.2
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	9p13-p12
PLEKHC1	mitogen inducible 2	14q22.1
HK2	hexokinase 2	2p13
CDK7	cyclin-dependent kinase 7	5q12.1
PPP6C	Homo sapiens protein phosphatase 6, catalytic subunit	9q34.11
MAPK7	mitogen-activated protein kinase 7	17p11.2
SRPK1	SFRS protein kinase 1	6p21.3-p21.2
CDC16	CDC16 cell division cycle 16 homolog	13q34
S100A6	S100 calcium binding protein A6	1q21
PLAGL1	pleiomorphic adenoma gene-like 1	6q24-q25
CPR8	cell cycle progression 8 protein	
ASNS	asparagine synthetase	7q21.3
QSCN6	quiescin Q6	1q24
EXT1	exostoses (multiple) 1	8q24.11-q24.13
MARS	DNA-damage-inducible transcript 3	12q13.1-q13.2
CDC16	CDC16 cell division cycle 16 homolog (S. cerevisiae)	13q34
CHFR	checkpoint with forkhead and ring finger domains	12q24.33
PTEN	phosphatase and tensin homolog	10q23.3
GAK	cyclin G associated kinase	4p16
RPS4X	ribosomal protein S4, X-linked	Xq13.1
RFP2	ret finger protein 2	13q14
FH	fumarate hydratase	1q42.1
AXL	AXL receptor tyrosine kinase	19q13.1
STAT1	signal transducer and activator of transcription 1, 91kDa	2q32.2
CPR8	cell cycle progression 8 protein	15q21.1
TCF7L2	transcription factor 7-like 2	10q25.3
ANKHD1	FLJ20288 protein	5q31.3
FHIT	fragile histidine triad gene	3p14.2
BIN1	bridging integrator 1	2q14

ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)	11q22-q23
NHP2L1	NHP2 non-histone chromosome protein 2-like 1	22q13.2-q13.31
BCL2	B-cell CLL/lymphoma 2	18q21.3
PNUTL2	peanut-like 2 (Drosophila)	17q22-q23
BTG2	BTG family, member 2	1q32
KIAA1009	KIAA1009 protein	6q15
CDC10	CDC10 cell division cycle 10 homolog	7p14.3-p14.1
PARD6A	par-6 partitioning defective 6 homolog alpha	16q21
CUL3	cullin 3	2q36.3
RAN	RAN, member RAS oncogene family	6p21
HDAC4	histone deacetylase 4	2q37.2
	Homo sapiens p58/GTA protein kinase mRNA, complete cds.	
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	1p32
GAS7	growth arrest-specific 7	17p
RGC32	RGC32 protein	13q13.3
CSK	c-src tyrosine kinase	15q23-q25
CDC7	CDC7 cell division cycle 7-like 1 (S. cerevisiae)	1p22
RALGDS	ral guanine nucleotide dissociation stimulator	9q34.3
CEB1	cyclin-E binding protein 1	4q22.1-q23
ANKHD1	FLJ20288 protein	5q31.3
CCND3	cyclin D3	6p21
ANKRD15	kidney ankyrin repeat-containing protein	9p24.3
PCAF	p300/CBP-associated factor	3p24
TERF2	telomeric repeat binding factor 2	16q22.1
CTCF	CCCTC-binding factor (zinc finger protein)	16q21-q22.3
TERF1	telomeric repeat binding factor (NIMA-interacting) 1	8q13
CDK2AP1	CDK2-associated protein 1	12q24.31
RGS2	regulator of G-protein signalling 2, 24kDa	1q31
RALGDS	ral guanine nucleotide dissociation stimulator	9q34.3
AIF1	allograft inflammatory factor 1	6p21.3
NME1	non-metastatic cells 1, protein expressed in	17q21.3
MCM5	MCM5 minichromosome maintenance deficient 5	22q13.1
DUSP6	dual specificity phosphatase 6	12q22-q23
CCND2	cyclin D2	12p13
RB1	retinoblastoma 1 (including osteosarcoma)	13q14.2
GPR21	rab6 GTPase activating protein	9q34.11
RBBP6	retinoblastoma binding protein 6	16p12-p11.2
GSPT1	G1 to S phase transition 1	16p13.1
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1p13.2
PCNA	proliferating cell nuclear antigen	20pter-p12
MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	3q21
MCM6	MCM6 minichromosome maintenance deficient 6	2q21
CCNB2	cyclin B2	15q21.2
MELK	maternal embryonic leucine zipper kinase	9p13.1
CCNB1	cyclin B1	5q12
UBE2C	ubiquitin-conjugating enzyme E2C	20q13.11
MAD2L1	MAD2 mitotic arrest deficient-like 1	4q27
F2R	coagulation factor II (thrombin) receptor	5q13
CDK2	cyclin-dependent kinase 2	12q13
CHEK1	CHK1 checkpoint homolog (S. pombe)	11q24-q24
CENPF	centromere protein F, 350/400ka (mitosin)	1q32-q41
HIRA	HIR histone cell cycle regulation defective homolog A	22q11.21
CDC5L	Homo sapiens KIAA0432 mRNA, partial cds.	6p21
PPAT	phosphoribosyl pyrophosphate amidotransferase	4q12
PKMYT1	tyrosine- and threonine-specific cdc2-inhibitory kinase	16p13.3
MCC	mutated in colorectal cancers	5q21-q22
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	8q11
TOPBP1	topoisomerase (DNA) II binding protein	3q22.1
RAD21	RAD21 homolog (S. pombe)	8q24
RBBP8	retinoblastoma binding protein 8	18q11.2
GMNN	geminin, DNA replication inhibitor	6p22.1
ORC5L	origin recognition complex, subunit 5-like (yeast)	7q22.1
MCM3	MCM3 minichromosome maintenance deficient 3	6p12
MCM7	MCM7 minichromosome maintenance deficient 7	7q21.3-q22.1
CDC10	CDC10 cell division cycle 10 homolog	7p14.3-p14.1
PDGFA	platelet-derived growth factor alpha polypeptide	7p22
UBE2V2	ubiquitin-conjugating enzyme E2 variant 2	8q11.1

APPENDIX IV

Presentations and pending publications

Awards

I was awarded additional funding in the 3rd year of my PhD from the Arthur and Mildred Slater Award UCL.

Conference Presentations

Queen Square Symposium – Institute of Neurology, 12th March 2004

- Poster presentation – Identification of aberrantly expressed genes in different grades of paediatric astrocytoma.

British Neuro-Oncology Society Conference – Telford, 23rd-25th June 2004

- Poster presentation – Identification of aberrantly expressed genes in different grades of paediatric astrocytoma.
  - Awarded runner up for best scientific presentation.

Queen Square Symposium - Institute of Neurology, 15th March 2005

- Poster presentation - Reduced survival of paediatric patients with atypical low grade astrocytoma is associated with a gain in chromosome 7q.
  - Awarded top 8 position for scientific presentation.

British Neurosurgical Research Group Meeting – NHNN, London, 17th March 2005

- Oral presentation - Reduced survival of paediatric patients with atypical low grade astrocytoma is associated with a gain in chromosome 7q.

World Federation of Neuro-Oncology Conference – Edinburgh, 4th-8th May 2005

- Poster presentation – Genes that promote cell growth are aberrantly expressed in pilocytic astrocytoma. The abstract was published in Neuro-oncology.

Queen Square Symposium - Institute of Neurology, 14th March 2006

- Poster presentation - Down-regulation of *PRDM2, SPINT2, DAPK1, CCNA1, REPRIMO* and *CDKN1C* is not caused by promoter hypermethylation in paediatric pilocytic astrocytoma.

British Neuro-oncology Society Meeting – Institute of Child Health London, 21st -23rd June 2006.

- Poster presentation - Down-regulation of *PRDM2, SPINT2, DAPK1, CCNA1, REPRIMO* and *CDKN1C* is not caused by promoter hypermethylation in paediatric pilocytic astrocytoma.
- Oral presentation - Identification of aberrantly expressed genes in different grades of malignancy in paediatric astrocytoma.

Society for Neuro-oncology – Orlando Florida, 16th -19th November 2006.

- Poster presentation - Abnormal gene expression can be linked to chromosome gains and losses in a number of paediatric astrocytomas.

Pending Publications

As a result of the work I have completed during my PhD I have 1 manuscript that has been accepted for publication in Neoplasia entitled:

- Genomic deletions correlate with under-expression of novel candidate genes at 6 loci in paediatric pilocytic astrocytoma

I also have another manuscript in preparation for journal submission entitled:

- Abnormal gene expression can be linked to chromosome gains and losses in paediatric astrocytoma.

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To my parents, Kay and Alan, I could not ask for two more supportive parents. During the time I have stayed in education you have offered nothing less than anything and everything. I thank you from the bottom of my heart and I hope I can repay you one day.

I would like to say a big thank you to those who have endured me on a daily basis, Blanca, Roy, Sam and Katherine. Without your technical advice and guidance I would probably still be in the lab now! I owe a special thank you to Katherine, for the many hours of deliberation and pondering we have had over various topics and conundrums.

To the ladies on the fifth floor, Gita, Sarah, Jo and Anouska, what can I say but that you have kept me sane! The camaraderie between us has been fantastic and kept me going during the tedious times.

To my sister Jayne, forever describing my thesis as 'brains in a petri dish'. A most inappropriate description but you have kept a smile on my face and I am grateful for that. Thank you my sweet.

To Lisa Wells, where do I start, we have supported each other throughout the last 4 years (and several before), over many bottles of wine and hours of conversation. It has been worth it in the end and in theory the world is now our oyster! Thank you.

Christopher Wall (a.k.a. Boomer) my source of constant enthusiasm and big hugs. When frustration and tiredness have loomed, you have given me perspective and drive, thank you gorgeous.

Oliver King, may you rest in peace. I want to thank you for giving me the courage to even contemplate a PhD in the beginning and possibly for the ridiculous nick-name of Dr Sausage.